MAST CELLS AND SYNOVIAL FIBROBLASTS AS NOVEL TARGETS FOR ANTIRHEUMATIC THERAPY

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Cover: Synovial fibroblasts isolated from tissue of a patient with rheumatoid arthritis and immunohistochemically stained with antibodies for Thy-1.

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Mast cells and synovial fibroblasts
as novel targets for antirheumatic therapy

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ACADEMIC DISSERTATION

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ORIGINAL PUBLICATIONS

This thesis is based on the following research papers, which are referred to in the text as their Roman numerals. Some unpublished data are presented.

I Sandler C, Ekokoski E, Lindstedt KA, Vainio PJ, Sorsa T, Kovanen PT, Golub LM, Eklund KK. Chemically modified tetracycline (CMT)–3 inhibits histamine release and cytokine production in mast cells: possible involvement of protein kinase C. *Inflamm Res. 2005;54:304-312.*

II Sandler C, Nurmi K, Lindstedt KA, Sorsa T, Golub L, Kovanen PT, Eklund KK. Chemically modified tetracyclines induce apoptosis in cultured mast cells. *Int Immunopharmacol (In Press)*

III Juurikivi A*, Sandler C*, Lindstedt KA, Kovanen PT, Juutilainen T, Leskinen M, Mäki T, Eklund KK. Inhibition of c-kit tyrosine kinase by imatinib mesylate induces apoptosis in mast cells in rheumatoid synovia; a potential approach for treatment of arthritis. *Ann Rheum Dis (In Press) *equally contributed*

IV Sandler C, Joutsiniemi S, Lindstedt KA, Kolah J, Juutilainen T, Kovanen PT, Eklund KK. Selective activation of mast cells in rheumatoid synovial tissue results in production of TNF-α, IL-1β and IL-1Ra. *(submitted)*

V Sandler C, Joutsiniemi S, Lindstedt KA, Juutilainen T, Kovanen PT, Eklund KK. Imatinib mesylate inhibits platelet derived growth factor stimulated proliferation of rheumatoid synovial fibroblasts. *(submitted)*

LIST OF ABBREVIATIONS

CML	chronic myeloid leukemia
CMT	chemically modified tetracycline
DMARD	disease-modifying antirheumatic drug
ELISA	enzyme-linked immunosorbent assay
FcRI	high-affinity IgE-receptor
FCS	fetal calf serum
GAPDH	glyseraldehyde-2-phosphate dehydrogenase
GIST	gastrointestinal stromal tumor
GM-CSF	granulocyte-monocyte colony stimulating factor
HMC-1	human mast cell line -1
HuMC	human CD34+ stem cell derived mast cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
LT	leukotriene
mBMMC	mouse bone marrow derived mast cell
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
PAF	platelet-activating factor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PG	prostaglandin
PKC	protein kinase C
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor-κB ligand
RANTES	regulated upon activation T-cell expressed and secreted
RPMC	rat peritoneal mast cell
SCF	stem cell factor
SFB	synovial fibroblast
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end-labelling
VEGF	vascular endothelial growth factor

ABSTRACT

Sandler, Charlotta 2005. *Mast cells and synovial fibroblasts as novel targets for antirheumatic therapy.*

Rheumatoid arthritis is a chronic destructive disease of joints associated with severe individual suffering. Despite the recent progress in the treatment of RA, a significant proportion of patients fail to respond to the current drug therapy reflecting the need for new drug innovations. In order to develop new therapies for RA, it is essential to increase the understanding of the mechanisms that contribute to the disease process. This study was designed to investigate whether mast cells and synovial fibroblasts could serve as novel targets for antirheumatic therapy.

Proinflammatory mediators produced by variety of cell types in diseased synovium play a central role in the pathogenesis of RA. Mast cells are capable of secreting an impressive array of proinflammatory mediators. The results of the present study show that selective activation of mast cells in synovial tissue obtained from patients with RA, results in a significant production of TNF-α and IL-1β which are the key cytokines in the pathogenesis of RA. These results support the previous observations that mast cells are involved in the pathogenesis of RA and thereby may provide a novel strategy for therapeutic intervention in RA.

Chemically modified tetracyclines (CMTs) are doxycycline derivatives that have the anticollagenolytic properties but lack the antibiotic properties of doxycycline. The results of the present study indicate that CMTs inhibits the cytokine production by cultured mast cells. Furthermore, CMT-3 inhibited cytokine production induced by IgE-dependent mast cell activation in rheumatoid synovial tissue. This inhibition was assigned to the observed ability of CMT-3 to inhibit the activity of protein kinase C. Finally, the results of the study showed that CMTs induce apoptosis in cultured mast cells.

Imatinib mesylate blocks the function of specific tyrosine kinases, including c-kit and platelet-derived growth factor (PDGF) receptor. Since signalling through these receptors is implicated in RA, this study aimed at investigating the effects of imatinib mesylate on the viability of mast cells and on the proliferation of synovial fibroblasts. The results show that imatinib mesylate induced a profound apoptosis in mast cells. Imatinib also inhibited significantly the proliferation of synovial fibroblasts which was attributed to its ability to prevent PDGF-mediated growth stimulation. Thus, imatinib mesylate represents a potential novel approach as it may exert antirheumatic activity by inhibiting mast cell activity and proliferation of synovial fibroblasts in rheumatoid synovium. The preliminary clinical findings on the efficacy of imatinib mesylate in the treatment of RA are consistent with this conclusion.

In conclusion, synovial mast cells seem to be capable of elaborating a significant inflammatory reaction in rheumatoid synovial tissue. The results of the study also show that CMT-3 and imatinib mesylate are effective inhibitors of mast cell function. Thus, these compounds might provide new approaches for the treatment of rheumatoid arthritis.

1.
INTRODUCTION

Rheumatoid arthritis (RA) is the most common inflammatory arthritis worldwide, with prevalence of 0.8 % in Finland. RA is a systemic inflammatory disease of unknown cause with a central feature of chronic inflammation in the synovium of the joints. Despite the impressive progress in the treatment of RA, the disease is associated with severe long-term outcomes and it is a major cause of disability. The pathogenesis of RA is still incompletely understood despite of intensive research. Nevertheless, many processes contributing to the pathogenesis have been uncovered.

RA is a chronic inflammatory disease. The underlying cause of RA is unknown. The disease is characterised by infiltration of inflammatory cells, such as T cells, macrophages and mast cells, into the synovium. The infiltrating cells produce abundant number of cytokines, such as TNF-α and IL-1β, which stimulate further recruitment and activation of inflammatory cells and resident synovial fibroblasts. These interactions lead to chronic inflammation in the joint, to fibroblast hyperproliferation and to formation of an invasive pannus tissue, ultimately resulting in the destruction of the joint structures.

Mast cells are powerful cells of the immune system that reside mainly in connective tissue and in mucosal membranes. Upon activation via the high-affinity IgE-receptors, mast cells secrete a wide array of proinflammatory mediators, most notably histamine, proteases, cytokines and arachidonic acid metabolites. However, mature mast cells are activated also via various IgE-independent mechanisms. A pathogenic role for mast cells is implicated in many inflammatory diseases such as allergic disorders and asthma. Increasing evidence suggests that mast cells play an important role also in chronic inflammatory disorders.

Recent research findings have strongly indicated that mast cells are involved in the pathogenesis of RA. Mast cell hyperplasia is prominent in inflamed synovium. Increased mast cell numbers correlate with the activity of the disease. Signs of mast cell activation have been detected both in RA synovium and in synovial fluid from patients with RA. However, lack of efficient and selective inhibitors of mast cell function has hampered the definitive clarification of the role of mast cells in RA.

The treatment of RA has improved significantly during the last decade. The recommended early and aggressive introduction of disease-modifying antirheumatic drugs (DMARDs) has improved the prognosis of new RA patients. The anticytokine therapies have revolutionised the treatment of severe RA. However, no curative treatments are available and a considerable proportion of patients do not respond

adequately to the current medications. Therefore, research contributions are needed to find new therapeutic approaches to treat the disease.

Imatinib mesylate is a tyrosine kinase inhibitor with relative selectivity towards bcr-abl tyrosine kinase, platelet-derived growth factor receptor (PDGFR) tyrosine kinase and c-kit receptor tyrosine kinase. A pilot clinical trial observed an antirheumatic effect in patients treated with imatinib (Eklund and Joensuu 2003), which could theoretically be explained by inhibition of mast cells via c-kit receptor and inhibition of synovial fibroblasts via PDGFR. Tetracyclines are a group of antibiotic agents that might hold therapeutical effects in treatment of RA and osteoarthritis (Stone et al. 2002). Chemically modified tetracyclines (CMTs) are doxycycline derivatives that act anticollagenolytic but lack the antibiotic properties. CMT-3 has been studied particularly in treatment of cancers.

In the present study, the role of mast cell activation in rheumatoid synovial tissue has been studied. The emphasis has been to determine the effects of two drugs, imatinib mesylate and chemically modified tetracycline-3, on the function of cultured and synovial tissue mast cells. In addition, the effect of imatinib on the proliferation of rheumatoid synovial fibroblasts was studied. These studies aimed at finding new means to inhibit mast cells and determining whether preventing mast cell activity and inhibiting the growth of synovial fibroblasts could serve as new approaches for the treatment of RA.

2.
REVIEW OF THE LITERATURE

2.1 MAST CELLS

Mast cells are granulated immune cells that are widely distributed throughout the body. They are derived from bone marrow progenitor cells that migrate into tissues, where they undergo final maturation. Proliferation, differentiation, survival and activation of human mast cells are regulated by stem cell factor (SCF), the ligand for the c-kit tyrosine kinase receptor which is expressed on the mast cell surface. Mast cells release a broad array of proinflammatory and immunoregulatory mediators after activation induced by either IgE-dependent or IgE-independent mechanisms. Mast cells have been most widely understood in the context of allergic reactions and parasite infections, but they are now also acknowledged as important players in innate and acquired immunity, wound healing, fibrosis, and chronic inflammatory diseases, such as RA.

2.1.1 Origin and maturation

Mast cells are derived from hematopoietic CD34+ stem cells that originate in bone marrow (Kirshenbaum et al. 1991). Mast cell progenitors are agranular mononuclear cells that are commited mast cell progenitors i.e. they can not differentiate to any other cell type (Rodewald et al. 1996). Progenitor cells circulate in peripheral blood before entering the tissues where they complete their differentiation and maturation to mast cells (Li et al. 1999). Circulating mast cell progenitors express c-kit receptors and low affinity FcγRII-receptors on their surface (Rodewald et al. 1996). In opposite to mature mast cells, the progenitor cells do not express high affinity FcεRI-receptors (Rodewald et al. 1996). These mast cell progenitors represent a proportion of 5-15% of total mast cell numbers in tissues, which indicate that mast cell progenitors migrate from blood to target tissues at an early stage of maturation (Craig et al. 1989).

The maturation of mast cells occurs in tissues. The key regulators of differentiation of human mast cells are SCF and its tyrosine kinase receptor c-kit (Valent et al. 1992, Mitsui et al. 1993, Irani et al. 1992). SCF is an essential factor for both mast cell maturation and maintenance of mature mast cells in the body, since it induces mast cell proliferation and suppresses mast cell apoptosis (Kirshenbaum et al. 1992,

Mekori et al. 1995). The critical role of SCF in mast cell maturation is supported by the findings that the number of tissue mast cells is greatly decreased in SCF-deficient mice (Sl/Sl^d) (Kitamura et al. 1978, Huang et al. 1990, Brannan et al. 1991) and in mice which lack the functional SCF receptor (W/W^v) (Kitamura and Go 1979, Geissler et al. 1988). In tissues, SCF is provided either in a soluble form or membrane-bound on fibroblasts (Kambe et al. 2001, Hogaboam et al. 1998), stromal cells (Galli et al. 1993) or endothelial cells (Mierke et al. 2000). In synovium, SCF is expressed by synovial lining cells, stromal fibroblasts, macrophages, endothelial cells, and in vascular basement membranes (Ceponis et al. 1998). In addition to regulation of mast cell viability, SCF acts as a chemoattractant for mast cells (Meininger et al. 1992, Kiener et al. 2000).

After the maturation process mast cells express FcεRI-receptors on their cell membrane and their cytosol is crowded by secretory granules (Kirshenbaum et al. 1991). In addition to maturation process of mast cell progenitors, mast cell number in tissues can increase due to local proliferation of mature mast cells in response to SCF (Kambe et al. 2001, Bischoff et al. 1999). In brief, mast cell populations in tissues are controlled by three different factors: recruitment of committed progenitors from circulation, maturation of resident progenitors in tissues, local proliferation of mature mast cells and apoptosis of tissue mast cells.

2.1.2 Distribution and heterogeneity

Mast cells are resident cells in tissues throughout the body. They are frequently found around blood vessels, in the vicinity of nerves, in the skin and at mucosal surfaces both in the airways and the intestine. Their location at the interface of host and environment reflects their role in early defence against invading pathogens (Metcalfe et al. 1997, Galli et al. 1999, Nathan 2002, Marshall 2004). Mast cells are found also in synovial tissue and their number has been shown to be increased under inflammatory conditions (Crisp et al. 1984b, Gotis-Graham and McNeil 1997). Under normal conditions mature mast cells are not found in peripheral blood (Metcalfe et al. 1997).

Mast cell populations are heterogeneous with variability in size, granule contents, cytokine production and receptor expression. Two major mast cell types have been identified in humans, the TC-type (MCTC) containing both tryptase and chymase, and the T-type (MCT) containing only tryptase (Irani et al. 1986, Irani et al. 1989). MCTC also contain cathepsin G (Schechter et al. 1990) and carboxypeptidase-A (Irani et al. 1991) in their granules. The local microenvironment is able to regulate the mast cell phenotype (Austen and Boyce 2001). The different mast cell subsets are found in different locations within human tissues. Furthermore, the distribution of mast cell subsets within tissues is altered in different disease stages.

In addition to SCF, mast cell migration and differentiation is influenced by several locally produced cytokines, such as IL-3 (Rottem et al. 1994, Ochi et

al. 1999), IL-4 (Toru et al. 1998, Bischoff et al. 1999), IL-9 (Ochi et al. 1999) and the nerve growth factor (Kanbe et al. 2000). The ability of SCF to support certain stages of mast cell differentiation is profoundly affected by interactions with other cytokines, particularly IL-4 and IL-10 (Rennick et al. 1995). Thus, the final differentiation of mast cell progenitors is dependent on the cytokine patterns that are present in different microenvironments.
In RA synovium, increased numbers of mast cells are frequently found close to the synovial lining layer and around blood vessels. MCTC is the major phenotype in healthy human synovial tissue, while expansion of the MCT subset is observed in early RA (Gotis-Graham et al. 1998). An increased density of MCT cells have been described in late RA synovium, but this was associated with a substantial expansion of MCTC cells (Gotis-Graham and McNeil 1997). Generally, it appears that the MCT subset is of importance in the inflammatory events in both early and late RA, whereas the MCTC subset may contribute to the repair of damaged connective tissue in late RA (Gotis-Graham and McNeil 1997, Gotis-Graham et al. 1998).

The two mast cell subsets show functional differences. In addition to their effects on mast cell differentiation and maturation, cytokines, such as SCF, IL-4, IL-5 and IL-6, can also modify the mediator release (Tkaczyk and Gilfillan 2001). MCTC and MCT also express distinct cytokine profiles. MCT have been found to produce predominantly IL-5 and IL-6, whereas MCTC in the same tissue produce IL-4 only (Bradding et al. 1995). In summary, the microenvironment around the mast cell regulates the phenotypic characteristics, receptor expression and the array of secreted mediators.

2.1.3 Mast cell activation

A fundamental feature of mast cells is their ability to secrete the contents of preformed cytoplasmic secretory granules in response to certain stimulants. Classically, crosslinking of the IgE, which is bound to high-affinity IgE receptors (FcεRI) on mast cells initiates signalling cascades leading to degranulation. Apart from activation via FcεRI, mast cells are also activated by cross-linking of surface FcγRI, by complement fractions C3a and C5a, by a range of small peptides, including substance P, and by chemokines, such as macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant peptide (MCP)-1.

Mature mast cells express FcεRI. The receptor is comprised of four subunits, an α subunit, a ß subunit and two γ subunits, which form a covalently linked homodimer. All the subunits lack intrinsic enzymatic activity. The FcεRI α subunit is responsible for the high-affinity binding of IgE, whereas the primary function of the FcεRI ß and FcεRI γ subunits is to recruit signalling molecules into the receptor. Binding of a multivalent antigen to FcεRIα/IgE complex provokes the IgE crosslinking and leads to aggregation of FcεRI α chains (Blank et al. 1989, Turner and Kinet 1999). The aggregation of FcεRI initiates the activation of intracellular coupled tyrosine

kinases, Lyn and Syk, which leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) and eventually to activation of different proteins and lipids, including phospholipase C isoforms, and protein kinase C (PKC), that are implicated in these signalling cascades (Jouvin et al. 1994, Metcalfe et al. 1997, Reischl et al. 1999, Turner and Kinet 1999). These cascades couple to further downstream steps, such as transient increase in free cytosolic [Ca^{2+}], and to additional activation of PKC. The signalling events finally culminate in the secretory response within seconds and minutes after the initial stimulation, and later to the production of cytokines. An overview of FcεRI-mediated signalling is presented in Figure 1.

Besides IgE-dependent activation, various other mechanisms exist that stimulate mast cells to degranulate. Mast cells express receptors for IgG. FcγRIIb and FcγRIIIa are low-affinity IgG-receptors expressed by mast cells and principally involved in the response to immune complexes (Nigrovic and Lee 2005). Following interferon (IFN) -γ treatment, human mast cells express also the high-affinity receptor for IgG, FcγRI (Okayama et al. 2000). Aggregation of these receptors in response to antigen binding results in mast cell degranulation. The signalling pathways activated by FcγRI aggregation generally resemble those activated via FcεRI. Also the mediator profile resembles that observed in human mast cells after IgE-dependent degranulation, although there are some differences in the profile of cytokine production (reviewed in Tkaczyk et al. 2004).

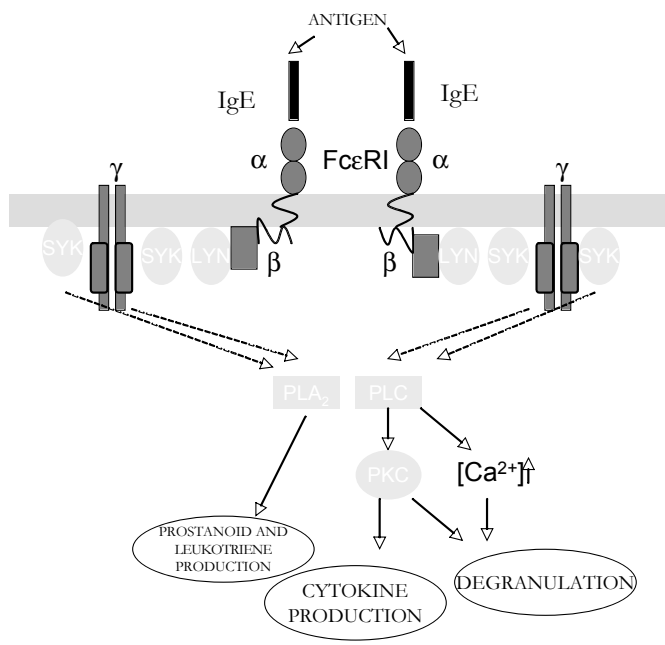

Figure 1. FcεRI-mediated intracellular signalling in mast cell. A simplified scheme of signaling pathways induced by antigen binding to FcεRI (modified from Tkaczyk and Gilfillan 2001).

The anaphylatoxic complement peptides C3a and C5a, have long been considered as mast cell activators (Metcalfe et al. 1981). However, it appears that only MCTC type mast cells express receptors for C3a and C5a, and can subsequently be activated by these complement components (Mousli et al. 1994). Synovial mast cells in patients with RA represent predominantly MCTC type and they have been shown to express C5a receptor and respond to stimulation with C5a (Kiener et al. 1998).

Mast cells have been found to be activated in various T cell-mediated inflammatory processes. Mast cells reside in close physical proximity to T cells in inflamed allergic tissues and at sites of parasitic infections (Mekori and Metcalfe 1999, Mekori and Baram 2002). T cells can mediate mast cell activation both by secreting soluble cytokines and by direct cell-cell-contact (reviewed in Mekori and Baram 2002). Conversely, activated mast cells produce mediators, such as leukotrienes, platelet-activating factor (PAF), TNF-α and macrophage inflammatory protein (MIP) -2, that affect T cell-mediated inflammatory reactions and recruit T-cells to the site of inflammation (Galli 1993, Biedermann et al. 2000, Ott et al. 2003). This interplay indicates an important function of mast cells within T cell-mediated inflammatory processes, which are strongly implicated in the pathogenesis of RA.

2.1.4 Mediators derived from mast cells

Mast cell activation has three major outcomes: explosive degranulation, with the release of preformed mediators such as proteases and histamine, ensuing generation of arachidonic acid derivatives, and in the later phase synthesis and secretion of cytokines and chemokines. However, all these events do not necessarily happen always, as certain mast cell activators induce only cytokine secretion without preceding degranulation. Typically, mast cell mediators have more than one function, and the mediators overlap in their biological effects. In this chapter, the mast cell mediators are reviewed with a special reference to joint inflammation. Mediators secreted from activated mast cells and examples of their physiological functions are listed in Table 1.

Mast cells are the major source of histamine in tissues (Metcalfe et al. 1997). Histamine is stored in mast cell secretory granules linked to the carboxyl groups of proteins and proteoglycans at acidic pH. Histamine is released within seconds after mast cell activation and rapidly metabolized in tissues. Thus, histamine usually influences events near the site of its release. Histamine H_1 and H_2 receptors mediate the wide-ranging biological activities of histamine. As a potent vasoactive and bronchoconstrictive agent, histamine is the central mediator of acute allergic and anaphylactic reactions. Histamine might contribute to the pathogenesis of arthritis as it stimulates MMP production by chondrocytes and by synovial fibroblasts (Zenmyo et al. 1995, Tetlow and Woolley 2002 and 2004).

Tryptase and chymase are the major proteins in mast cells, which emphasise their significance in mast cell function. Up to 30 % of the mast cell granule protein may consist of these neutral proteases (Welle 1997). Both mast cell proteases appear

to be involved in inflammatory reactions. Mast-cell-derived tryptase has been shown to stimulate fibroblast proliferation (Levi-Schaffer and Piliponsky 2003, Frungieri et al. 2005), which is one of the cornerstones in the pathogenesis of RA. Chymase converts a precursor of IL-lβ to an active form (Welle 1997). Moreover, mast cell tryptase and chymase have been shown to activate in vitro the zymogen forms of the matrix metalloproteinases (Gruber et al. 1988a, Saarinen et al. 1994), which is likely to play a role in the cartilage destruction associated with RA.

Within minutes after encountering stimuli, mast cells begin to generate lipid mediators from cell membrane arachidonic acid (Murakami et al. 1995). These include, depending on the circumstances, PGD_2, LTC_4, LTB_4 and platelet-activating factor (PAF). LTB_4 increases vascular permeability. Mast-cell-derived LTB_4 appears to be involved in the triggering of T cell migration to the site of inflammation, which might play a role in synovitis (Ott et al. 2003). PGD_2 is a vasoactive mediator and acts as a chemoattractant for neutrophils (McInnes 2003). However, the PGD_2 metabolite 15-d-PGJ_2 is emerging as an anti-inflammatory mediator (Harris 2002). It has been shown to induce synoviocyte apoptosis and to suppress experimental arthritis (Kawahito et al. 2000).

Mast cell has the capacity to produce an exceptional wide array of cytokines, including TNF-α, TGF-β, GM-CSF, SCF, IL-1, IL-4, IL-6, IL-8, IL-13, IL-16, MCP-1 and MIP-2. The assortment of produced cytokines depends on the mast cell type and stimulus. Mast cells are the only cell type that can store preformed TNF-α in granules and release it quickly upon activation (Gordon and Galli 1990). Mast cells also produce large amounts of TNF-α in response to activation. TNF-α is a multifunctional cytokine vitally involved in numerous physiological and pathophysiological processes (reviewed in Pfeffer 2003 and in Ghezzi and Cerami 2004). The main function of TNF-α appears to be the promotion of inflammation. TNF-α causes activation and differentiation of macrophages and monocytes. Moreover, TNF-α promotes inflammation by inducing the production of other cytokines, such as IL-1β, and also MMP and prostaglandins (Dayer et al. 1985). TNF-α is a chemotactic molecule (Kharazmi et al. 1988). TNF-α also increases vascular permeability and induces upregulation of cellular adhesion molecules ICAM-1 (intracellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule) in endothelial cells (Osborn 1990, Walsh et al. 1991). Furthermore, TNF-α induces production of other chemokines by a variety of cells (Strieter et al. 1989, Butler et al. 1995). Thus, TNF-α generates a multidimensional positive signal for leukocyte migration to the site of inflammation. In addition, TNF-α is a potent inducer of apoptosis. The cytotoxic effect is mediated via TRAIL (tumor necrosis factor related apoptosis-inducing ligand), which selectively induces apoptosis in a variety of tumor cells and transformed cells, but not in most normal cells (Wang and El-Deiry 2003). The role of TNF-α in the pathogenesis of RA is undisputable and is discussed later in this review (see 2.2.2).

Class	Mediators	Examples of function
Granule-associated	histamine serotonine	alter vascular permeability
	heparin and chondroitin sulphate	enhance cytokine function and angiogenesis
	tryptase, chymase, carboxypeptidase, and other proteases	remodel tissue and recuit effector cells
	TNF-α, VEGF, FGF2	recruit effector cells and enhance angiogenesis
Lipid-derived	LTC4, LTB4, PGD2, PGE2	recruit effector cells regulate immune responses promote angiogenesis oedema bronchoconstriction
	Platelet activating factor	enhances angiogenesis, induces inflammation
Cytokines	TNF-α IL-1α IL-1β IL-6 IL-18, GM-CSF, LIF, interferons	induce inflammation
	IL-3, IL-4, IL-5, IL-9, IL-13, IL-15, IL-16	T-cell helper 2-type cytokines
	IL-12, IFN-γ	T-cell helper 1-type cytokines
	IL-10 TGF-β VEGF	regulate inflammation and angiogenesis
Chemokines	CCL 2, CCL3, CCL4, CCL5, CCL11, CCL20	recruit effector cells, regulate immune responses
	CXCL1, CXCL2,CXCL8, CXCL9, CXCL10, CXCL11	recruit effector cells, regulate immune responses
Others	nitric oxide and superoxide radicals	bactericidal, proinflammatory
	antimicrobial peptides	bactericidal

Table 1. Mediators released by activated mast cells (modified from Marshall 2004).
TNF=tumor necrosis factor, VEGF=vascular endothelial factor, FGF=fibroblast growth factor, LT=leukotriene, PG=prostaglandin, IL=interleukin, GM-CSF=granulocyte macrophage-colony stimulating factor, LIF=leukemia inhibitory factor, IFN=interferon, TGF=transforming growth factor, CCL, CXCL=chemokine families

In summary, mast cells are equipped with variety of potent inflammatory mediators. Mediators produced by activated mast cells contribute to inflammatory processes. Mast cells exert also chemotactic signals and promote the recruitment of leukocytes to the site of inflammation.

2.1.5 Mast cells in health and disease

Mast cells have been conserved in evolution, despite of their detrimental role for the host in allergic diseases and anaphylaxis, suggesting there have to be benefits to the host. The critical role of mast cells in host defence against bacterial infections has been clearly demonstrated (reviewed in Marshall 2004). The importance of IgE-mediated mast cell activation to the health is incompletely understood. One likely advantage of it is the resistance to parasitic diseases (Marshall 2004). The appropriate release of mediators by mast cells challenged with pathogens has clear protective effects to the host. However, the excessive or inappropriate release of inflammatory mediators from mast cells causes pathological symptoms in many common diseases.

The remarkable variety of proinflammatory mediators derived from mast cells, and the recognition that mast cells release these mediators in response to stimulation by many different signals in addition to IgE-mediated stimulation, provides theoretical basis to the involvement of mast cells in multiple diseases associated with inflammation. Furthermore, the expansion of local mast cell numbers and the evidence of degranulation have been observed in a wide spectrum of pathological responses and in a large number of diseases, including asthma (Hart 2001, Robinson 2004), atherosclerosis (Kaartinen et al. 1998, Lindstedt and Kovanen 2004), fibrosis (Gruber 2003, Levi-Schaffer and Piliponsky 2003), autoimmune diseases (Benoist and Mathis 2002), and inflammatory bowel diseases (He 2004). Also mast cell neoplasias, including cutaneous and systemic mastocytosis and mast cell leukemia, exist (Valent et al. 2005). Mast cells are today also implicated in rheumatoid arthritis, which is discussed elsewhere in this review (see chapter 2.3).

Mast cells are implicated in numerous diseases where acute and/or chronic inflammation is known to play a role. Typically, mast cell numbers increase in diseased tissues due to augmented migration of mast cell progenitors and/or reduced apoptosis of mature tissue mast cells. Thereby intervention with drugs that inhibit mast cell activity or induce apoptosis of mast cells could be potentially beneficial in many common diseases, like allergies, asthma, atherosclerosis and inflammatory arthritis.

2.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is the most common inflammatory arthritis. RA occurs in 0.5–1.0 % of the adult population worldwide, with prevalence of 0.8 % in Finland (Kaipiainen-Seppänen and Aho 2000). There is a genetic predisposition of the disease, with clearly defined involvement of HLA of DR4/DR1 (Stasny 1978). Of an unknown reason RA is 2–3 times more common in females than in males (Sangha 2000). The mean age at diagnosis of RA is 59.0 years in Finland (Kaipiainen-Seppänen and Aho 2000). The onset of the disease has moved to older age classes. Also the severity of the disease has decreased during the last 20 years. The reasons for these changes are unknown. Despite the impressive progress in the treatment of RA, the disease is still associated with severe long-term outcomes, including declines in functional status, increased work disability, and increased mortality (Scott et al. 1987, Weinblatt 2003, Pincus et al. 2004).

2.2.1 Pathogenesis

RA is a systemic inflammatory disease of unknown cause. A central feature of RA is the chronic inflammation in the synovium of the joints. The disease is characterised by a chronic, symmetrical progressive polyarthritis that typically affects synovial joints. The natural history of RA varies from self-limited, nonerosive to severe, destructive disease. The first symptoms are typically pain, stiffness and swelling in the small joints of fingers and toes. RA may have systemic manifestations including fatigue, low-grade fever, vasculitis, anemia, osteoporosis, and secondary amyloidosis.

The pathogenesis of RA is still incompletely understood despite of intensive research. The typical pathological changes in synovial membrane of RA patients are hyperplasia, increased vascularity and infiltration of inflammatory cells. CD4+ T cell is the main inflammatory cell type in inflamed synovium and it is considered as the main orchestrator of cell-mediated immune responses. One supposed initiator of the inflammation in RA is an unidentified antigen, which activates CD4+ T cells. The antigen could be either an exogenous antigen, such as viral protein, or an endogenous protein, such as collagen. Antigen-activated CD4+ T cells in turn stimulate monocytes, macrophages and synovial fibroblasts to produce cytokines and to secrete MMPs. The stimulation occurs via direct cell-surface contact or by release of soluble mediators. Activated CD4+ T cells can also activate B cells through cell-surface signalling to produce immunoglobulins, including rheumatoid factor. The precise pathogenic role of rheumatoid factor is unknown, but it may involve the activation of complement through the formation of immune complexes (reviewed in Feldmann 2001 and in Firestein 2003).

Joint damage occurs progressively in patients with RA. The proinflammatory cytokines IL-1β and TNF-α play a key role in promoting cartilage and bone erosions in the inflamed joint. The appearance of structural damage in joints is

an indicator of disease severity and can be used to predict the future disability. Activated macrophages and synovial fibroblasts produce MMPs that degrade extracellular matrix, and cytokines and proangiogenic factors that contribute to the inflammation and to the formation of vasculature. Endothelial cells in the synovium are activated and they express adhesion molecules, such as ICAM-1, that promote the recruitment of inflammatory cells into the joint. This process is enhanced by the release of chemokines by inflammatory cells in the joint.

2.2.2 Cytokines in RA

The term cytokine is applied to a rapidly growing group of small proteins or glycoproteins that serve as chemical messengers between cells. Cytokines are involved in critical processes as cell growth and differentiation, tissue remodelling, and regulation of the immune response. In acute and chronic inflammation cytokines play a key role in regulating the nature, magnitude and duration of the inflammatory response. Most cytokines are expressed transiently after an inducing stimulus. One of the most potent signals for inducing the production of cytokines is other cytokines. Thus, cytokines create a network, in which they interact in synergistic, complementary, antagonistic or inhibitory manner. The dysregulation of this cytokine network is an important feature of the pathology of RA, and as such, cytokines represent an important therapeutic target for antirheumatic treatment.

There is considerable evidence that TNF-α and IL-1β are the key mediators that drive inflammation and tissue degradation in RA. As illustrated in Figure 2,

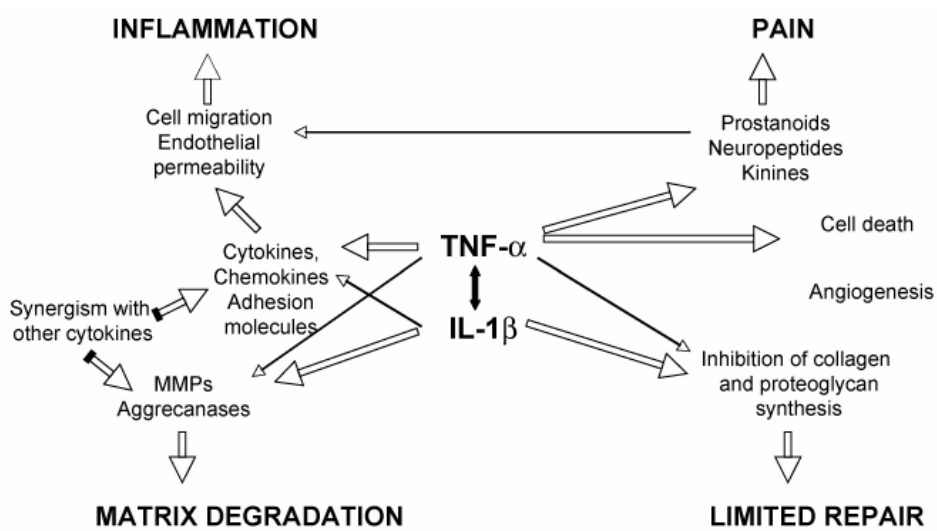

Figure 2. IL-1β and TNF-α are the key mediators that drive inflammation and tissue degradation in RA (modified from Dayer 2003).

both cytokines influence local and systemic disease manifestations in a synergistic manner. Also numerous other cytokines have been implicated in RA and an overview of them is presented in Table 2. In this review, the role of TNF-α, IL-1β and IL-1Ra is discussed in more detail.

The apprehension that TNF-α is one of the most important factors in the pathogenesis of RA was first realized approximately 15 years ago. In 1988, Di Giovine et al. detected that TNF-α levels are increased in synovial fluid and serum of RA

Cytokine	Function	Effect
IL-2	stimulates T-cell proliferation	proinflammatory
IL-6	induction of immunoglobulin production in B cells activation of T cells proliferation of synovial fibroblasts induction of acute phase response	proinflammatory
	down-regulation of TNF- and IL-1 induction of synthesis of TIMP	anti-inflammatory
IL-7	expression of adhesion molecules stimulates immunoglobulin synthesis	proinflammatory
IL-8	induces neutrophil infiltration and activation stimulates angiogenesis	proinflammatory
IL-15	recruitment, migration and activation of T cells inhibition of T cell apoptosis induction of production of IL-8 and MCP-1	proinflammatory
IL-4	inhibition of T cell activation inhibition of TNF-, IL-1, IL-6 and IL-8 production increase in IL-1Ra production	anti-inflammatory
IL-10	inhibition of the production TNF- and IL-1 inhibition of the T cell proliferation	anti-inflammatory
IL-18	production of TNF- and IL-1 expression of adhesion molecules	proinflammatory
IL-11	decrease in TNF- and IL-1	anti-inflammatory
IL-13	decrease in TNF- , IL-1, PgE2 and NO increase in IL-1Ra	anti-inflammatory

Table 2. Pro- and anti-inflammatory cytokines in rheumatoid arthritis (reviewed in Choy and Panayi 2001).

IL=interleukine, TNF=tumor necrosis factor, TIMP=tissue inhibitor of metalloproteinases, MCP=monocyte chemoattractant protein, PG=prostaglandin, NO=nitric oxide

patients. TNF-α expression was also shown to be increased in the inflamed joints of animals with experimentally induced arthritis and in patients with RA (Firestein et al. 1990). Transgenic mice overexpressing TNF-α were found to spontaneously develop an erosive arthritis (Keffer et al. 1991). Furthermore, several investigators noticed that administration of TNF-α exacerbated arthritis and inhibitors of TNF-α reduced the severity of inflammation (Cooper et al. 1992, Piquet et al. 1992, Williams et al. 1992). These findings led to years of intense research culminating in the introduction of anti-TNF-α therapies for RA in the beginning of 21st century.

TNF-α is a soluble 17 kD protein composed of three identical subunits. TNF-α is bound to cell membrane until cleaved by TNF-α converting enzyme (TACE) (Black et al. 1997). Biological responses to TNF-α are mediated via two structurally distinct receptors, type I (TNFRI) and type II (TNFRII). Both receptors are expressed on all cell types except erythrocytes. Both TNF-α receptors are subject to proteolytic cleavage and are shed from the cell surface in response to inflammatory signals. The shed receptor retains its ability to bind TNF-α, thus acting as a natural inhibitor of TNF-α activity. Both soluble inhibitors are readily detectable in rheumatoid synovial fluids, but in concentrations insufficient to neutralize endogenous production of TNF-α (Cope et al. 1992, Steiner et al. 1995). The expression of TNF-α receptors is upregulated in an active RA tissue (Alsalameh et al. 1999) and TNF-α receptors are expressed by a variety of cell types in RA synovial tissue, indicating that abundant cells are potential targets for TNF-α (Deleuran et al. 1992).

While TNF-α is physiologically vital in activating the innate and acquired immune response against pathogens, it is obvious that inappropriate production of TNF-α leads to inflammation and tissue destruction. TNF-α is produced by numerous cell types including immune cells (B cells, T cells, basophils, eosinophils, monocytes, macrophages, dendritic cells, NK cells, neutrophils and mast cells), nonimmune cells (such as fibroblasts, osteoclasts and smooth muscle cells) and generally tumour cells (Bazzoni and Beutler 1996, Pfeffer 2003, Ghezzi and Cerami 2004). Immunohistochemical studies of RA synovium have localized TNF-α predominantly in macrophages (Chu et al. 1991, Tetlow and Woolley 1995a). However, TNF-α has also been found to be expressed by synovial mast cells in RA tissues (Woolley and Tetlow 2000).

TNF-α alone is not especially arthritogenic, as antibodies against IL-1 receptor completely arrest the arthritis in TNF-α transgenic mice model of chronic arthritis, even if the TNF-α levels reside high (Probert et al. 1995, van den Berg 2002). The power of TNF-α is that it is the prime cytokine stimulating and co-ordinating the overall cytokine response. This provides a rationale for therapeutic targeting. The finding by Brennan et al. (1989), that blocking TNF-α leads to a significant decrease in the production of IL-1β, was the cornerstone for the development of anti-TNF-α therapy for treatment of RA. The concept was further strengthened by the findings that blocking TNF-α reduces the expression of also multiple other proinflammatory cytokines in synovial tissue, such as granulocyte-monocyte colony

stimulating factor (GM-CSF) (Haworth et al. 1991), IL-6 and IL-8 (Butler et al. 1995). Anti-TNF-α based therapies have proven highly effective in the treatment of RA, furthermore confirming the role of TNF-α in the pathogenesis of RA (Elliott et al. 1993 and 1994, Feldmann and Maini 2001). Also the hypothesis, that TNF-α orchestrates the cytokine response, has been confirmed in vivo by the findings that levels of IL-6 (Charles et al. 1999), VEGF (Paleolog et al. 1998), IL-8 and other chemokines (Taylor et al. 2000) are decreased in response to anti-TNF-α treatment in patients with RA.

When the general interest on cytokines in the pathophysiology of RA greatly increased in late 80's to early 90's, also IL-1β emerged as a key contributor in the disease. Patients with active RA were shown to have elevated IL-1β concentrations in plasma (Fontana et al. 1982, Eastgate et al. 1988). The level of IL-1β in serum was found to generally correlate with the disease severity (Cannon et al. 1993). Injection of IL-1 into the knee joints of rabbits was shown to give rise to the degradation of cartilage (Pettipher et al. 1986). Furthermore, the administration of antibodies to IL-1 completely prevented the development of arthritis in TNF-transgenic mouse model (Probert et al. 1995). IL-1β is involved in several central pathological processes related to RA as it stimulates the expression of proinflammatory mediators, tissue-damaging enzymes and antiapoptotic molecules in synovial fibroblasts (Jeong et al. 2004). IL-1β expression tends to be increased in end stage of RA, suggesting a major role of IL-1β in joint destruction (Smeets et al. 2003). The last line of evidence for the pivotal role of IL-1 in RA comes from studies showing that specific blocking of IL-1β reduces the intensity and destructive nature of the disease (van den Berg et al. 1994, Campion et al. 1996, Bresnihan et al. 1998, Bendele et al. 1999).

The interleukin-1 family consists of seven individual members. Of these, three members, IL-1α, IL-1β and IL-1Ra are thoroughly studied in the context of RA. IL-1β binds to two types of cell surface receptors. Only IL-1RI has a cytoplasmic tail and is capable of evoking intracellular signal (Sims et al. 1993). IL-1RII is a decoy receptor, as it binds IL-1 but do not deliver any intracellular signal (Colotta et al. 1993). Both IL-1 receptor types are found also in soluble form. Soluble forms compete with cell-surface receptors, thus decreasing the biological activity of IL-1. In addition, a naturally occurring antagonist, IL-1Ra, binds to IL-1RI without triggering a signal (Svenson et al. 1995). Thus, the biological activity of IL-1 is physiologically regulated by three different mechanisms. Similarly to TNF-α, IL-1β is produced by macrophages and monocytes, but also other cell types like endothelial cells, synovial lining cells, B cells and activated T cells (Koch et al. 1995). In immunohistochemical studies on rheumatoid synovial tissue, IL-1β has been shown to be expressed in cells neighbouring mast cells (Woolley and Tetlow 2000).

TNF-α and IL-1β have a significant overlap in their biological effects and their synergism is marked concerning inflammation and tissue remodeling. In general, TNF-α seems to be more important at the systemic level and in the inflammatory context, while IL-1β acts at the local level and is central in the development of erosions. It is

evident that TNF-α induces the production of IL-1β and vice versa (Nawroth et al. 1986, Dinarello et al. 1986, Phillip and Epstein 1986, Probert et al. 1995). Practically all cells express receptors for TNF-α and therefore respond to it. IL-1β induces the production of proteolytic enzymes, such as MMPs, and activates osteoclasts, both effects playing a key role in the development of cartilage and joint erosions.

IL-1Ra is a naturally occurring antagonist of IL-1. It binds to IL-1R with high affinity without evoking a signal thus inhibiting the binding of agonistic IL-1. The inhibitory function of IL-1Ra appears to be unique in cytokine biology. Neutrophils and mononuclear cells are the main sources of IL-1Ra (Malyak et al. 1993, Firestein et al. 1994), but also rheumatoid synovial fibroblasts have been shown to produce IL-1Ra (Chomarat et al. 1995). The importance of IL-1Ra in counteracting the destructive effects of IL-1β was shown in mice deficient in the IL-1Ra gene, which spontaneously developed arthritis and erosions of the articular bone (Horai et al. 2000).

The relative production of IL-1β to IL-1Ra is fundamental in the pathophysiology of RA. The concentrations of IL-1Ra are high in the synovial fluid of RA patients, but not high enough to bind all the excess IL-1β for suppression of inflammation (Chomarat et al. 1995). Less than 5% of IL-1 receptors need to be engaged by IL-1β in order to induce a biological response, and it is estimated that a 10- to 100-fold excess of IL-1Ra is necessary to achieve a 50% inhibition of the IL-1 response (Arend et al. 1990). Thus, the reported elevation of IL-1Ra in rheumatoid synovial fluids appears insufficient to abolish IL-1β-induced inflammatory responses.

2.2.3 MMPs in RA

The matrix metalloproteinases (MMPs) are a large group of enzymes responsible for matrix degradation and turnover in both physiology and pathology. They contribute to joint destruction in RA by directly degrading the cartilage and bone and indirectly by promoting angiogenesis. The imbalance between MMP expression, activation, and inhibition is implicated in RA (Murphy et al. 2002, Mohammed et al. 2003, Firestein 2003, Tchetverikov et al. 2004). Aggrecanase and collagen are the major components of the joint cartilage that are subjects to proteolytic degradation by MMPs. MMP-1 (collagenase-1), MMP-13 (collagenase-3) and MMP-3 (stromelysin-1) are presumed to play central roles in the cartilage destruction associated with rheumatoid arthritis and their expression has been shown to be markedly increased in the synovium of patients with active RA (Konttinen et al. 1999a).
In rheumatoid synovium, synovial fibroblasts and chondrocytes are the major sources of MMPs. TNF-α and especially IL-1β are known to induce MMP expression in SFB (Dayer et al. 1985, Kumkumian et al. 1989). Furthermore, these cytokines have been detected at the same areas as the increased MMP activity in the RA tissue (Konttinen et al. 1999b), signifying the role of this induction in RA.

2.3 MAST CELLS IN RA

The role of mast cells in inflammatory arthritis has been discussed since 1980´s (Wasserman 1984, Crisp 1984a, Mican and Metcalfe 1990). The observed mast cell hyperplasia in inflamed synovium and the exceptionally broad panel of potent proinflammatory effectors released by activated mast cells gave rise to the concept of mast cell being a forceful player in synovial inflammation. Furthermore, the comprehension that mast cells are activated by immune complexes and complement fragments strenghthened the hypothesis of their involvement in synovitis. A recent breakthrough, describing the mast cells as a key cell type in RA animal model (Lee et al. 2002), has again made the conversation lively around the subject (Woolley 2003, Nigrovic and Lee 2005). To date, it has become evident that mast cells are involved in the pathogenesis of RA. Figure 3 sums up the dimensions discussed in this chapter.

2.3.1 Mast cell hyperplasia

An increased density of mast cells in RA synovium is a consistent finding (Wyenne-Roberts et al. 1978, Godfrey et al. 1984, Crisp et al. 1984b, Gotis-Graham and McNeil 1997). It has been estimated that mast cells normally constitute

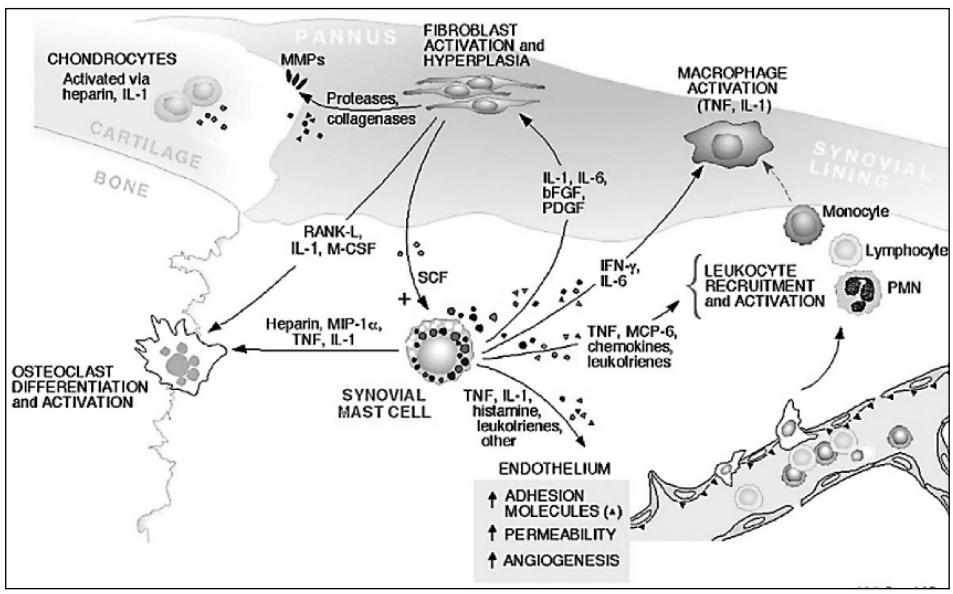

Figure 3. Mast cell in inflammatory arthritis.
(Arthritis Res Ther. 2005;7:1-11, with permission of the copyright holder).
TNF=tumor necrosis factor, IL=interleukin, MCP=monocyte chemoattractant protein, PMN=polymorphonuclear cells, IFN=interferon, bFGF=basic fibroblast growth factor, PDGF=platelet-derived growth factor, SCF=stem cell factor, MMP=matrix metalloproteinase, RANK-L=receptor activator of NF-kappaB-ligand , M-CSF=macrophage-colony stimulating factor, MIP=macrophage inflammatory protein.

approximately 3 % of all cells within synovium, and that this proportion is increased up to 5 % or more in RA synovium (Nigrovic and Lee 2005). The degree of mast cell hyperplasia in synovial tissues seems to be related to the degree of clinical synovitis, the extent of lymphocytic infiltration and the activity of RA (Malone et al. 1987, Gotis-Graham and McNeil 1997, Gotis-Graham et al. 1998). Increased SCF levels have been detected in synovial fluid of RA patients (Carsons et al. 2000), and increased infiltration and maturation of mast cell progenitors appear to be the main reasons for hyperplasia in synovium (Ceponis et al. 1998). Mast cells are present throughout the synovial sublining with accumulations in pannus near sites of erosions (Tetlow and Wooley 1995a and 1995b). Mast cells are also present in the synovial fluid of patients with RA. Synovial fluids from patients with RA have been shown to exhibit mast cell chemotactic activity, which might be one explanation for the observed mast cell hyperplasia (Olsson et al. 2001). As activation of mast cells induces expression of antiapoptotic molecules (Xiang et al. 2001, Möller et al. 2003), an additional contributing factor might be reduced apoptosis of mast cells in RA synovium. Besides RA, synovial mastocytosis has been observed also in other arthritides, including juvenile RA, systemic lupus erythematosus, psoriatic arthritis, and osteoarthritis (Godfrey et al. 1984).

2.3.2 Mast cells and chronic inflammation

Accompanying the increased number of mast cells, mast-cell-specific mediators are elevated in the synovial fluid of RA patients indicating continuing mast cell activation (Malone et al. 1986, Buckley et al. 1997). The role of mast cells in inflammation is twofold. They contribute directly to the production of inflammatory cytokines but probably even more important is their capacity to recruit and stimulate other effector cells.

The greatly expanded number of inflammatory cells is a prominent feature of RA synovium. Mast cells induce expression of adhesion molecules on endothelial cells (Walsh et al. 1991) and secrete an array of chemotactic mediators thus recruiting neutrophils and lymphocytes (Kharazmi et al. 1988, Strieter et al. 1989). Furthermore mast cell mediators increase vascular permeability thus facilitating the passage of inflammatory cells.

Macrophages and T-cells are well-known for their contribution to synovitis related to RA (reviewed in Firestein 2003). Mast-cell-derived mediators, such as TNF-α, IL-6, GM-SCF and MCP-6 activate macrophages and T cells and thus upholding the chronic inflammation in the joint.

2.3.3 Mast cells and angiogenesis

Formation of new blood vessels is a characteristic feature of inflamed synovium. Angiogenesis begins very early in the course of RA. Angiogenesis ensures development

and persistence of the pannus by increasing the supply of oxygen, nutrients, cytokines, and inflammatory cells to the synovial membrane. Clinical disease severity in RA patients is correlated with the intensity of angiogenesis, as evaluated by VEGF levels or other parameters (Clavel et al. 2003). In several studies, VEGF levels have been found to be higher in synovial fluid or blood from RA patients than from controls (Fava et al. 1994, Koch et al. 1994, Paleolog et al. 1998). Furthermore, VEGF levels were correlated with clinical and laboratory markers for disease activity and progression in patients with RA (reviewed in Clavel et al. 2003).

Fibroblasts and endothelial cells are the main sources of proangiogenetic cytokines, including PDGF, VEGF and bFGF. However, in synovium, mast cells reside closely associated with blood vessels and are found at sites of angiogenesis (Norrby 2002). Mast cells exert proangiogenic effect as they produce several proangiogenic mediators, including VEGF, bFGF and IL-8 (Yamada et al. 1998, Qu et al. 1995, Norrby 2002). Furthermore, mast cell proteases, such as tryptase and MMPs, support the process by degrading connective tissue matrix to provide space for new capillaries. In addition to direct effects, mast cells indirectly stimulate angiogenesis by secreting TNF-α and IL-1β, both of which greatly enhance the expression of VEGF by other cell types (reviewed in Norrby 2002).

2.3.4 Mast cells and fibrosis

Mast cells are known to be a source of fibrogenic cytokines, including PDGF and bFGF (Qu et al. 1995, Artuc et al. 2002). In accordance with this mast cells have been shown to stimulate fibroblast proliferation (Trautmann et al. 1998). The supportive effect is bidirectional as SFB produce SCF, which is the main growth factor for mast cells, and thus stimulate mast cell chemotaxis and support mast cell survival in synovial tissue (Ceponis et al. 1998, Kiener et al. 2000). Interactions between primary pulmonary fibroblasts and mast cells have been shown to result in increased mast cell activity (Hogaboam et al. 1998). This induction was shown to depend on transmembrane SCF and require cell-cell contact. Given that both the fibrogenic cytokines and SCF are found to be elevated in RA, the bidirectional interaction between SFB and synovial mast cells is likely to contribute to the pathogenesis of RA. Similar two-way interactions have been detected also between macrophages and fibroblasts (Scott et al. 1997, Wang et al. 1997).

2.3.5 Mast cells and tissue degradation

The destruction of cartilage and bone is characteristic for RA and it is the ultimate reason for the loss of joint function. Mast cells secrete proteinases, such as tryptase, chymase and others, which directly degrade cartilage. Secondly, mast cells also have the potential to stimulate the expression of degrading enzymes, mainly MMPs, in neighbouring cells, including fibroblasts and chondrocytes. Simultaneously mast

cells also directly stimulate osteoclast differentiation and activation, which are mainly responsible for bone erosions in RA (Kobayashi et al. 2000). Furthermore mast cells attribute to osteoclast activity by inducing the production of osteoclast stimulating cytokines, such as RANK-L, by synovial fibroblasts (Gravallese et al. 2000, Nigrovic and Lee 2005).

In RA synovial tissue, mast cells are found at sites of cartilage erosions (Bromley et al. 1984) and mast cell activity has been associated with expression of MMPs in the tissue (Tetlow and Woolley 1995b). Mast cell activation has been shown to elevate MMP production in rheumatoid synovial tissue (Tetlow and Woolley 1995a, Tetlow et al. 1998). Mast cell tryptase and chymase activate in vitro the precursor forms of MMPs (Gruber et al. 1988a, Saarinen et al. 1994), further intensifying the effect of mast cells on tissue degradation. All this suggest that mast cells participate to the degradative processes within RA.

2.3.6 Mast cells in animal models of RA

In vitro mast cells exhibit several characteristics, which propose their involvement in arthritis in vivo. The role of mast cells in experimental arthritis has been investigated for over 30 years now (Gryfe et al. 1971, van den Broek et al. 1988). Quite recently, Lee et al. (2002) made a hallmark observation that mast cells are crucial for the development of erosive arthritis in response to autoantibodies in mice. Administration of serum from arthritic K/BχN mice induces arthritis in recipient mice (Korganow et al. 1999). However, the arthritogenic serum failed to induce arthritis in Sl/Sl^{d} or W/W^{v} murine strains, which lack functional mast cells. Importantly, engraftment of mast cells restored the susceptibility of W/W^{v} mice to arthritis. Lee et al. (2002) observed mast cell degranulation specifically in synovial tissue and it was related to synovial hyperplasia, cartilage and bone destruction and inflammatory filtrates. The signs of arthritis were absent in mast cell deficient mice.

2.3.7 Clinical evidence for the involvement of mast cells in RA

Clinical evidence for the involvement of mast cells in RA is limited. The research on this field has been hampered by the lack of efficient and selective inhibitors of mast cell functions. Administration of corticosteroids results in clinical improvement and can be accompanied by a decrease in mast cell numbers (Malone et al. 1987), but the effect of corticosteroids is naturally not mast cell specific. Recently, a drug that inhibits a key growth factor receptor expressed on mast cells, namely imatinib, has become available. The compound has been shown to inhibit growth of malignant mast cell line cells (Heindrich et al. 2000). Eklund and Joensuu (2003) have published a pilot clinical study with three RA patients, all of whom profited from imatinib during the 12 week period. In addition, Miyachi et al. (2003) observed a benefit in one RA patient treated with imatinib because of CML. A single patient with RA

at Helsinki University Central Hospital has been treated with imatinib for nearly 3 years and the activity of RA was very low during the entire treatment period. After 3 months of treatment with imatinib, synovial tissue of this patient contained only a few mast cells as judged by immunohistochemical analysis (unpublished data). Thus, eliminating synovial mast cells could offer benefit in RA indicating that these cells are involved in the disease process. However, whether the possible depletion of synovial mast cells is related to the antirheumatic effect of imatinib is currently not known.

2.4 SYNOVIAL FIBROBLASTS IN RA

The synovium is normally a relatively acellular structure with a delicate lining. The lining cells consist of macrophage-like (type A) synoviocytes and fibroblast-like (type B) synoviocytes. In RA, a marked hyperplasia of synovial fibroblasts (SFB) leads to thickening of the synovial membrane and to formation of pannus (Konttinen et al. 2000, Ritchlin 2000). The histological appearance of SFB in RA is characterized by expansion of endoplasmic reticulum and increase in the number of cytoplasmic granules, suggesting an increased synthetic activity (Smith et al. 1997, Pap et al. 2000). In the natural course of RA, the normally subtle synovial membrane is transformed into a proliferating invasive cell mass, consisting mostly of fibroblasts and macrophages, but also of mast cells, polymorphonuclear leukocytes, dendritic cells and plasma cells (Bromley and Woolley 1984).

The expanding pannus tissue erodes the surrounding articular tissue and bone. Current evidence shows that synovial cells act as the main effector cells in the joint destruction of RA through invading and degrading the cartilage tissue (Konttinen et al. 2000, Pap et al. 2000, Ritchlin 2000, Tolboom et al. 2002). This process destroys local articular structures, which is devastating to the joint function. Moreover, synovial cells are likely to have a fundamental role in upholding synovitis as they release chemokines, that recruit inflammatory cells to the joint, and cytokines that both elicit inflammation and trigger angiogenesis (Smith et al. 1997).

Inflammatory cells are present in rheumatoid synovium and they induce proliferation of SFB by releasing PDGF and other stimulating growth factors (Konttinen et al. 2000). Stimulated mast cells and macrophages produce large amounts of proinflammatory cytokines, in this case mainly TNF-α and IL-1β, which are potent inducers of proliferation and activation of SFB. IL-1β has been shown to provoke a series of responses in SFB including enhancement of inflammatory cytokines, imbalanced production of MMPs and tissue inhibitors of metalloproteinases (TIMPs), and dysregulation of apoptosis (Jeong et al. 2004), all strongly implicated in the pathology of RA. In the vicious circle of inflammatory arthritis, SFB further stimulate the recruitment inflammatory cells to synovium

(Smith et al. 1997, Kiener et al. 2000), thus triggering their own stimulatory signals.

The invasive growth of pannus is enabled by SFB capability to express degradative enzymes, MMPs in particular, that digest the extracellular matrix in the joint (Tolboom et al. 2002). SFB also stimulate the differentiation and activation of osteoclasts, resulting in bone erosion (Gravallese et al. 2000). Additionally, in response to stimulation, synovial cells produce proinflammatory cytokines that facilitate the persistence of chronic inflammation in the affected joint.

In the search for new effective treatments for RA, one important approach could be the inhibition of the overgrowth and the synthetic activity of SFB. Therefore understanding of the factors that attribute to stimulation of SFB is essential. In the chronically inflamed synovium, RA synovial fibroblasts grow aggressively under the continuous stimulatory signals. These cells have typically defective contact inhibition, which leads to tumour-like growth. Mutations in key growth regulating genes, like tumour suppression gene p53, have been suggested to be involved in RA synovial fibroblast proliferation (Pope 2002). Several anti-apoptotic molecules are expressed in SFB, and reduced rate of apoptosis is likely to be one reason for SFB hyperplasia in RA. Inhibition of proliferation and induction of apoptosis in SFB are considered as attractive therapeutic approaches in the treatment of RA (Pope 2002, Baier et al. 2003).

2.5 CHEMICALLY MODIFIED TETRACYCLINES

Tetracyclines are a group of antibiotic agents that have been proposed to hold therapeutical effects with subantimicrobial doses in treatment of RA and osteoarthritis (Ryan et al. 1996, Golub et al. 1998, Stone et al. 2003). However, in the long-term use, the antimicrobial properties of tetracyclines may evoke undesirable side effects, such as gastrointestinal disturbance and antibiotic resistance. Chemically modified tetracyclines (CMTs) are tetracycline derivates lacking the antibiotic activity of tetracyclines but possessing their anti-inflammatory properties.

Golub et al. (1987) found that removal of the dimethylamino group from the carbon-4 position of the A-ring of the 4-ringed tetracycline structure eliminates the antimicrobial activity while retaining the anticollagenolytic activity. This modification created CMT-1. Further modifications of CMT-1 have generated CMTs with more potent anti-collagenolytic activity, oral bioavailability and long serum half-life. Elimination of the methyl and the hydroxyl groups of CMT-1 resulted in CMT-3. To date more than 30 different CMTs have been synthesized and characterized. The chemical structures of CMT-1, CMT-3, CMT-8, CMT-308 and doxycycline are presented in Figure 4.

2.5.1 Effects of CMTs

Recent research has shown that the CMTs are pluripotent drugs that affect many cellular functions including proliferation, migration, and matrix remodeling. The most prominent effect of CMTs is the inhibition of matrix metalloproteinases (MMPs). The proposed mechanism of action of CMTs results from their ability to bind metal ions, particularly Zn^{2+} and Ca^{2+}, which are required by many enzymes to maintain their proper conformation and hydrolytic activity. CMT-3 inhibits effectively especially MMP-2 and MMP-9 (Acharya et al. 2004), but has also shown potency against MMP-1, MMP-3, MMP-8 and MMP-13 (Hanemaaijer et al. 1998, Golub et al. 1998).

Another interesting property of CMTs is their capacity to induce apoptosis. CMTs have been reported to prevent proliferation and to induce apoptosis in several malignant cell lines (D'Agostino et al. 2003, Lokeshwar et al. 2002, Tolomeo et al. 2001, Meng et al. 2000, Seftor et al. 1998). But CMT-3 has also been reported to induce apoptosis in some nonmalignant cultured cells, i.e. human osteoclasts (Holmes et al. 2004) and rat smooth muscle cells (Islam et al. 2003). The proapoptotic effect of CMTs possibly contributes to the observed anticancer efficacy of CMTs.

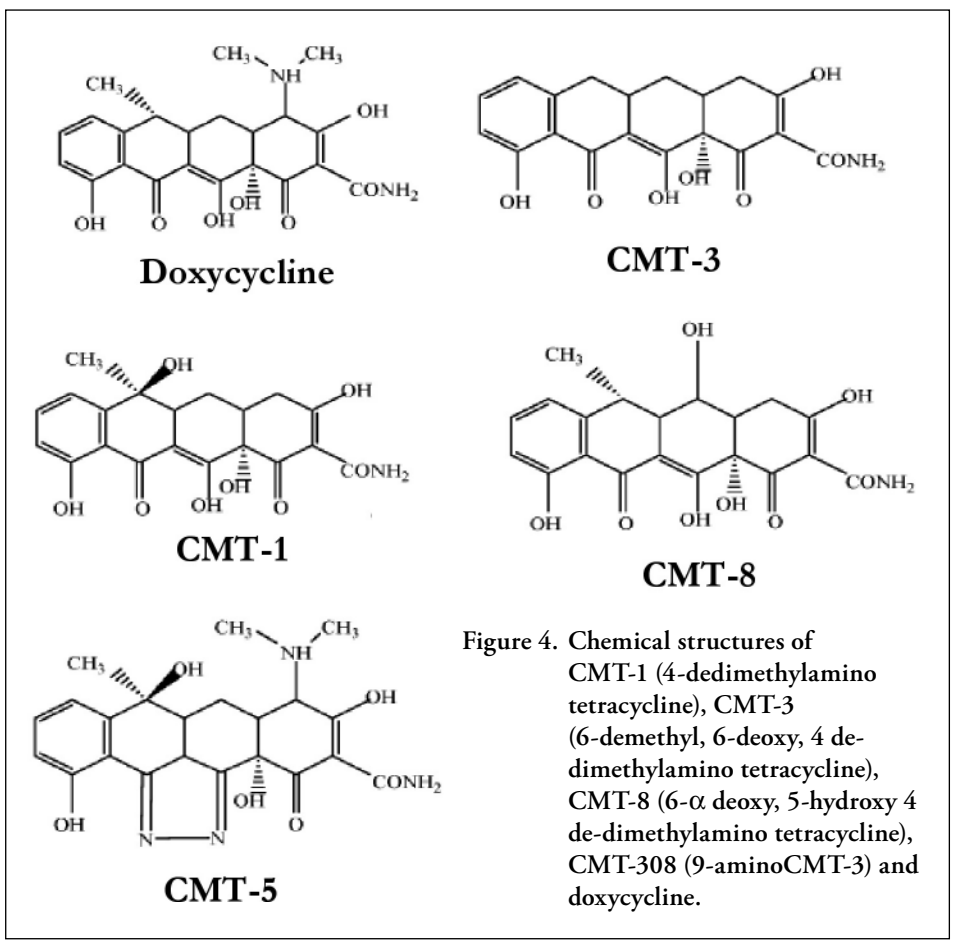

Figure 4. Chemical structures of CMT-1 (4-dedimethylamino tetracycline), CMT-3 (6-demethyl, 6-deoxy, 4 de-dimethylamino tetracycline), CMT-8 (6-α deoxy, 5-hydroxy 4 de-dimethylamino tetracycline), CMT-308 (9-aminoCMT-3) and doxycycline.

In addition, CMTs have been described to exert several anti-inflammatory characteristics, including inhibition of phospholipase A_2, inhibition of NO synthesis (D'Agostino et al. 2003, Tolomeo et al. 2001), inhibition of COX-2-mediated PGE_2 production (Patel et al. 1999) and inhibition of IL-6 formation (Kirkwood et al. 2003). The clinical significance of these findings is still to uncover.

2.5.2 CMT-3

CMT-3 (Metastat®, CollaGenex Pharmaceuticals, USA) is the first non-antimicrobial, chemically modified tetracycline assessed in clinical trials for anticancer effects in humans. On the basis of the promising preclinical studies (Cianfrocca et al. 2002, Rudek et al. 2001), CMT-3 is currently undergoing phase II trials for the potential treatment of metastatic cancer and HIV-related Kaposi's sarcoma. The results from this phase II clinical study indicate that CMT-3 potentially offers significant benefits for Kaposi's sarcoma patients (Press release 30.4.2004, CollaGenex Pharmaceuticals, USA). However, CMT-3 has shown efficacy also in other cancer diseases (Rudek et al. 2001, Syed et al. 2003).

The pharmacokinetics of CMT-3 in humans has been clarified (Rudek et al. 2001 and 2003, Cianfrocca et al. 2002, Syed et al. 2003). The main side effect of CMT-3, observed in 69 % of patients, is cutaneous photosensitivity. The frequency and severity of photosensitivity is dose-related and this side effect is the dose-limiting factor (Rudek et al. 2001, Syed et al. 2003). Nevertheless, biologically relevant concentrations are readily maintained at doses, which do not give rise to severe toxicity (Syed et al. 2003). The main mechanism regarding the antitumour activity of CMT-3 is believed to be the inhibitory effect on MMPs (Acharya et al. 2004). MMPs are implicated in tumour invasion and metastasis. In advantage, compared to other MMP inhibitors, CMT-3 also possesses antiproliferative and proapoptotic activity. Furthermore, CMT-3 has been shown *in vitro* to inhibit angiogenesis, an event that is critical for the growth of tumour (Fife et al. 2000).

2.6 IMATINIB MESYLATE

Imatinib mesylate (imatinib, Glivec® or Gleevec®, Novartis, Switzerland) represents a new generation of molecularly targeted therapies designed to interfere specific signal transduction pathways. Imatinib was formerly referred as STI571, and even in earlier studies known as CGP 57148B. The chemical structure of imatinib is presented in Figure 5.

2.6.1 Pharmacodynamic profile

Imatinib is a tyrosine kinase inhibitor with relatively selective activity against the bcr-abl tyrosine kinase, the abl-related gene ARG, platelet-derived growth factor receptor (PDGFR) and c-kit receptor (Druker et al. 1996, Buchdunger et al. 2000, Heinrich et al. 2000). Recently, imatinib has been shown to inhibit also the macrophage colony-stimulating factor (M-CSF) receptor, c-fms (Dewar et al. 2005).

Protein kinases are enzymes that transfer phosphate from adenosine triphosphate to specific amino acids on substrate protein. The phosphorylation of these proteins leads to the activation of signal-transduction pathways, which have a critical role in a variety of biological processes, including cell growth, differentiation and death. Protein kinases are composed of two subfamilies, the protein serine-threonine kinases and the protein tyrosine kinases (reviewed in Robinson et al. 2000). The deregulation of protein kinase activity plays a central role in the pathogenesis of human cancer and inhibition of protein kinases by selective pharmacological inhibitors is considered as an attractive therapeutic goal in treatment of cancers (Hanahan and Weinberg 2000).

Imatinib targets PDGFR (Buchdunger et al. 2000). Active PDGF molecules are dimeric isoforms that bind to two structurally similar tyrosine kinase receptors, PDGFR-alpha and PDGFR-beta. Because the PDGF isoforms are dimeric molecules,

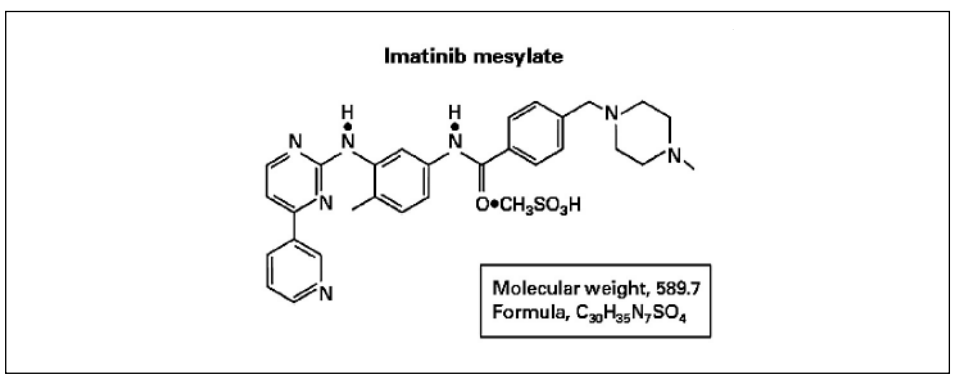

Figure 5. Chemical structure of imatinib mesylate.

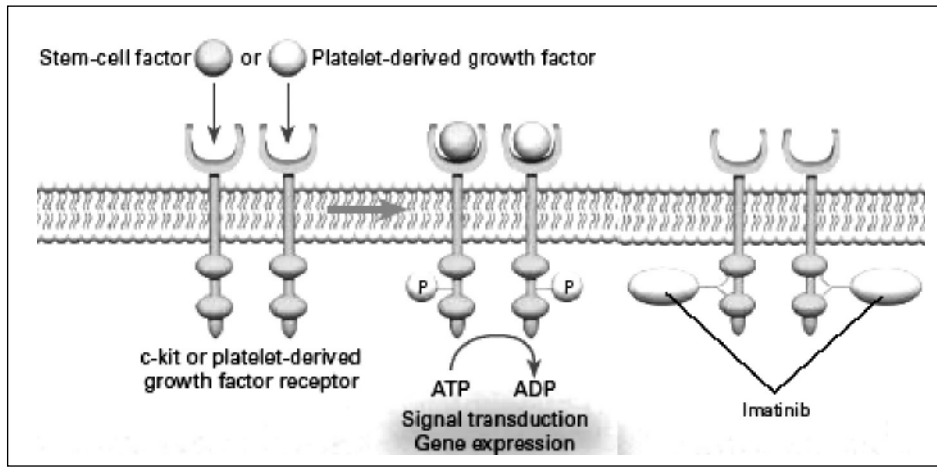

Figure 6. Imatinib mesylate inhibits the autophosphorylation of tyrosine kinase receptors for stem cell factor and platelet-derived growth factor. (N Engl J Med 2002;346:685, with permission of the copyright holder).
ATP=adenosine triphosphate, ADP=adenosine diphosphate, P=phoshphate

their binding to the PDGFR causes dimerization, leading to autophosphorylation and activation of the kinase activity of the receptor (Figure 6). The c-kit receptor that bind SCF, is structurally similar to PDGFR Imatinib blocks the PDGF and c-kit signalling pathway by inhibiting the receptor autophosphorylation (Savage and Antman 2002). Imatinib has been shown to inhibit the kinase activity of the members of type III receptor tyrosine kinase family, except flt 3 tyrosine kinase (Buchdunger et al. 2000, Dewar et al. 2005).

2.6.2 Clinical indications

Imatinib was approved by the Food and Drug Administration (USA) and European Medicines Agency (EU) in 2001 for the treatment of chronic myeloid leukemia (CML) and in 2002 for the treatment of gastrointestinal stromal tumors (GIST).
The Bcr-Abl tyrosine kinase is the molecular abnormality behind CML, which is a myeloproliferative disorder. The Bcr-Abl protein is a constitutively activated tyrosine kinase (Sawyers 1999, Faderl et al. 1999). Imatinib inhibits this activity and it has revolutionized the drug therapy of CML (Druker et al. 2001). The exceptional level of efficacy of imatinib is a result of precise targeting of the underlying cause of CML. Although a resistance to imatinib mesylate in CML patients have been observed, imatinib is at the moment a superior treatment for patients suffering from CML.

Mutated c-kit and PDGFR alpha tyrosine kinases are the principal targets for imatinib mesylate in the treatment of gastrointestinal stromal tumors. Most GISTs respond to imatinib mesylate, and it is the first treatment available for these patients. Imatinib is now considered as the standard systemic therapy for advanced GIST (Heinrich et al. 2003, Joensuu and Kindblom 2004).

2.6.3 Experimental findings

In addition to indications approved by the authorities, imatinib has shown therapeutical potency in several disorders. The unique mechanism of action of imatinib has stimulated intensive research on numerous fields of medicine.

Imatinib has shown to be effective against mast cell malignancies driven by mutated c-kit. Pardanani et al. (2003) reported the efficacy of imatinib in patients diagnosed with systemic mastocytosis. Half of these patients responded to imatinib therapy and 20 % of patients showed complete clinical and histological remission. In the perspective, that there is no current cure for mastocytosis, these results are noteworthy, and imatinib is considered useful in the treatment of systemic mastocytosis (Valent et al. 2005). However, relative resistance against imatinib exist, and it is associated with certain c-kit mutations (Valent et al. 2005).

2.7 TREATMENT OF RA

As the cause of RA is still elusive, no cure for the disease has been discovered. However, the treatment of RA has improved dramatically during the last 10 years. The disease-modifying antirheumatic drugs (DMARDs) improve signs and symptoms of the disease as well as slow down the disease progression. Early and aggressive intervention with DMARDs is recommended as soon as the diagnosis is confirmed. The introduction of biological therapies targeted against TNF-α and IL-1β, in late 1990's has begun a new era in the treatment of RA. In this review three antirheumatic treatments, methotrexate, TNF-α inhibitors and IL-1 receptor antagonist, are discussed in more detail. The latter two groups are also known as biological response modifiers (BRMs).

2.7.1 Disease-modifying antirheumatic drugs

DMARDs are drugs that besides reducing inflammation also slow the natural progress of the disease. There is strong evidence that destruction occurs already in the early course of RA (Pincus et al. 1995) and to date there is general agreement that DMARDs should be introduced as early as possible and with adequate force, to suppress disease activity and thus possibly avoiding further joint damage and disability (Emery and Salmon 1995, Lard et al. 2002, Möttönen et al. 2002, O'Dell 2002, Maillefert et al. 2003). The advantages of early and aggressive treatment of RA with DMARDs have been conclusively shown in Finnish patients in a 5 year follow-up study (Puolakka et al. 2004 and 2005, Korpela et al. 2005).

The effect of DMARDs is seen in1-3 months after the onset of the medication. When monotherapy fails to achieve or sustain clinical remission, DMARDs are typically used as combinations (O'Dell et al. 1996, Möttönen et al. 1999, Pincus

1999, Zerkak and Dougados 2004). Safety profiles of most DMARDs combinations are generally good (Zerkak and Dougados 2004). DMARDs currently in clinical use in Finland are listed in Table 3.

Methotrexate is considered as the first-in-line medication for RA and it is the most commonly used DMARD worldwide (Weinblatt 1995, Rau and Herborn 2004). In most of the cases, it also creates the optimal basis for combination therapy (Pincus et al. 2003). In clinical research, methotrexate has been accepted as the "gold standard treatment" and the new therapies are evaluated against it. As typical for most of DMARDs, the precise mechanism of antirheumatic effect of methotrexate is not completely understood. The classical mechanism of action of methotrexate is the inhibition of dihydrofolate reductase and other folate dependent enzymes leading to interference with the synthesis of pyrimidine and purines. Recently the ability of methotrexate to increase adenosine levels has been considered important to its antirheumatic efficacy (Rau and Herborn 2004). Low-dose methotrexate in RA treatment seems to exert anti-inflammatory effects by acting at different levels of the pathophysiological cascade (Cutolo and Straub 2001).

Sulphasalazine	Penicillamine
Methotrexate	Azathioprine
Hydroxychloroquine	Podophyllotoxin
Chloroquine	Cyclophosphamide
Cyclosporine	Infliximab
Aurothiomalate	Etanercept
Auranofin	Adalimumab
Chlorambucil	Anakinra

Table 3. Disease-modifying antirheumatic drugs currently used in Finland.

2.7.2 Biological response modifiers

To date, four biological treatments have been approved as therapies for RA. Infliximab is a chimeric (75 % human, 25 % mouse) IgG1 monoclonal antibody that neutralizes TNF-α. Adalimumab is a fully human monoclonal antibody to TNF-α. Etanercept is a soluble TNF-receptor fusion protein that binds to circulating TNF-α blocking the interaction with the cell-surface receptor. Etanercept can also bind to cell surface bound TNF-α. Treatment with agents that block the function of TNF-α has proved to be a highly effective approach in RA (Elliot et al. 1994 and 1995, Feldmann and Maini 2001). In addition to RA, these treatments have been effective in Crohn´s disease (infliximab), spondyloarthropaties, psoriasis, and graft-versus-host reactions (Reimold 2003).

Many of the pathophysiological processes involved in RA have been shown to be prevented by the use of TNF-α inhibitors. TNF-α blockers provide relief against

pain and joint swelling in most RA patients (van den Berg 2001). The immediate immunological mechanisms resulting from blocking of TNF-α appear to be crucial since clinical improvement is seen within days after the onset of treatment. However, anti-TNF-α therapy is not effective in all RA patients, indicating that multiple pathophysiological pathways exist.

Preventing erosions is central in antirheumatic therapy. The main mechanism for the erosive effect of TNF-α is assumed to be the upregulation of IL-1β production (van den Berg 2002). TNF-α has been also proposed to stimulate directly proliferation of synovial fibroblasts and differentiation of osteoclast (Kobayashi et al. 2000), which both are central in the development of bone erosions. The inhibition of these events could explain the antierosive effect of TNF-α inhibitors seen in RA patients (Bresnihan 2002a).

Another approach is to interfere the biological activity of IL-1β. Anakinra is a recombinant human IL-1 receptor antagonist. Anakinra binds to IL-1 receptors, thereby blocking the binding and biological effects of IL-1α and IL-1β (Arend et al. 1990). Anakinra has been shown to produce clinically significant improvements in signs and symptoms of RA and in the functional status of RA patients (Bresnihan 2002b). The therapeutic effects occur early and are sustained throughout treatment over 76 weeks. As IL-1β is the key cytokine in stimulating degradative processes associated with RA, anakinra has expectedly slowed the rate of joint erosions significantly in comparison to placebo (Bresnihan 2002b). The long-term effects of IL-1 blockage are still to uncover. In general, when comparing the efficacy of different biological, the ACR responses observed with anakinra are smaller than those observed with TNF-α blockers.

2.7.3 Future aspects of the treatment of RA

To date, no antirheumatic treatment has fully prevented the progression of joint damage in all patients with RA. Hence, there is a clinical need for new drug candidates. Several promising molecules are in the last phases of the drug development process and some new treatments for RA are expected in the near future.

The importance of CD4+ T cells in autoimmune diseases, such as RA, is clear. Abatacept (CTLA4-Ig) is a novel fusion protein designed to modulate T cell activation through blocking the classic T cell co-stimulatory signal (Ruderman and Pope 2005). Two completed phase III studies have proved abatacept to be effective in controlling the clinical signs and symptoms of RA (Mariette 2004, Ruderman and Pope 2005). Importantly, abatacept showed efficacy in patient with inadequate response to methotrexate or anti-TNF-α therapy.

As RA has an autoimmune background, also B cells play a role in the pathogenesis by producing autoantibodies, presenting antigens, and secreting cytokines. Rituximab is a chimeric monoclonal antibody for CD20 and targets mature B-cells.

It was originally designed for treatment of B cell leukemias. Rituximab has shown efficacy in phase II trials for treatment of RA (Edwards et al. 2004).

Interleukines are considered as potential targets in chronic destructive arthritis. IL-6 levels are elevated in serum and joint fluid from patients with RA, and a correlation has been found between serum IL-6 levels and the severity of radiographic destruction (Dasgupta et al. 1992). A humanized anti-IL-6R antibody called tocilizumab (or MRA) has recently been found effective in large phase II randomized placebo-controlled trials (Nishimoto et al. 2004). Blockade of IL-15 has also been tested with promising results in RA (McInnes and Gracie 2004).

New potential drug targets emerge as the basic research on the pathogenesis of RA advances. However, one can not predict which of the cytokines or cell types contributing to the chronic inflammation would be the best targets to interfere. Several animal models of RA are available, and the obtained results from these investigations may suggest novel targets. However, several treatments which have shown promising results in animal models, have later on failed in clinical trials (Keystone 2002), emphasizing the importance of preclinical research on human tissues. As the understanding of the multidimensional network of cytokines and cells participating in the development of chronic and erosive joint inflammation in RA evolves, the discovery of a cure for this detrimental disease may become closer.

3.
AIMS OF THE STUDY

The purpose of the present study was to investigate the role of mast cells in synovial inflammation and the ability of novel anti-inflammatory compounds to inhibit mast cell and synovial fibroblast functions. The effects of chemically modified tetracyclines and imatinib mesylate were studied in the present work.

This study was designed to test the following hypotheses:

1. The activation of synovial mast cells results in production of cytokines in rheumatoid synovial tissue (IV).
2. Chemically modified tetracycline-3 inhibits the activation of mast cells and ensuing secretion of mediators from mast cells (I).
3. Chemically modified tetracycline-3 inhibits the activity of protein kinase C (I).
4. Chemically modified tetracyclines induce apoptosis of mast cells (II).
5. Inhibition of c-kit signalling by imatinib mesylate induces apoptosis in mast cells (III).
6. The inhibition of PDGF receptor by imatinib mesylate inhibits the proliferation of rheumatoid synovial fibroblasts (V).

4.
MATERIALS AND METHODS

This chapter covers the materials and methods used in this thesis. A more detailed description of materials and methods is given in the respective original publications I-V.

4.1 Cell cultures

4.1.1 Human mast cell line

The human leukemic mast cell line (HMC-1) has been established from the peripheral blood of a patient with mast cell leukemia (Butterfield et al. 1988). HMC-1 cells were cultured in supplemented Iscove´s medium. HMC-1 cells were used in cytokine release (I), proliferation (II, III), apoptosis (II, III) and caspase activity (II, III) studies.

4.1.2 Rat peritoneal mast cells

Rat peritoneal mast cells (RPMC) were isolated from adult male Wistar rats (Test animal facilities, University of Helsinki, Finland). To collect the mucosal mast cells, thoracic and peritoneal cavities were lavaged with buffer. The buffer was centrifuged and cells were suspended into supplemented RPMI 1640 medium. The cells were incubated in a Petri dish for 1 h at +37 °C and non-adherent cells were collected and resuspended into fresh media. The purity of mast cells recovered was > 90 % as assessed by toluidine blue staining, which selectively stains mast cells. RPMC were used in histamine release (I, III) studies.

4.1.3 Mouse bone marrow derived mast cells

Mouse bone marrow derived mast cells (mBMMC) were isolated from 7-8 weeks old female BALB/c mice (Test animal facilities, University of Helsinki, Finland). Cells were harvested from bone marrow of femurs and tibias by repeated flushings with RPMI. The cells were cultured in IL-3 containing WEHI:RPMI medium. Non-adherent cells were transferred to fresh medium twice a week for 3-4 weeks to remove adherent macrophages and fibroblasts. The purity of mast cells derived as described has been reported to be relatively high (Eklund et al. 1994). mBMMC were used in proliferation (II, III) and apoptosis (II, III) studies.

mBMMC were cultured in IL-3 containing WEHI:RPMI (50:50) media or in SCF containing KL:RPMI (1:5) media. Cultured murine myelomonocytic leukemia cell line, WEHI-3 cells (line TIB-68, ATCC; Rockville, MD) produce IL-3 and release it in the media (Lee et al. 1982). WEHI-3 cells were cultured in supplemented RPMI media for 5-7 days. Then the filtered media was suspended with an equal amount of fresh supplemented RPMI. Recombinant c-kit ligand (rKL) was produced in Cos cells by transiently transfecting the cells by electrocorporation with artificially truncated KL cDNA (Flanagan et al. 1991). rKL containing Cos cell supernatant was used at dilution of 1:5 with supplemented RPMI corresponding approximately 150 ng/ml of rKL (Eklund et al. 1994). Before suspending cells cultured in IL-3 media, into rKL containing media the cells were washed three times with PBS to remove remaining IL-3.

4.1.4 Human mast cells obtained by culturing CD34+ stem cells

Human mast cells (HuMCs) were derived by culturing mononuclear CD34+ stem cells from cord blood, as described previously (Saito et al. 1995). The CD34+ cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10 % FCS. First (weeks 1–3) the cells were cultured in the presence of human recombinant SCF, IL-6 and IL-10. During the following 5 weeks (weeks 4–8), the cells were cultured in the presence of SCF, and thereafter in the presence of SCF and IL-4. For the first 10 weeks, the cells were cultured in a low-oxygen atmosphere (5% O2–95 % N2) and thereafter in a normal cell culture atmosphere (5 % CO2, ca. 20% O2). The maturity of cultured HuMCs was assessed by visualising the expression of tryptase and chymase and by determining histamine release. HuMCs were used in histamine release (I) and apoptosis (II and III) studies.

4.1.5 Human synovial fibroblasts

Human synovial fibroblasts (SFB) were isolated from synovial tissue obtained from knee arthroplasties of patients with diagnosed RA. All patients met the American College of Rheumatology classification criteria for RA (Arnett et al. 1987). The study was approved by the ethical committee of surgery of the Helsinki University Central Hospital. Synovial cells were dissociated by collagenase treatment and cultured in supplemented Dulbecco´s modified Eagles medium (DMEM). Before growing confluent cells were trypsinized and divided onto new cell culture plates. During the culture, macrophage-like synovial cells gradually disappear and fibroblast-like synovial cells proliferate. The phenotype of cultured cells was verifyied with immunohistochemical staining for fibroblast specific Thy-1 (see 4.9.2). The purity of SFB culture was approximately 90 %. Experiments were performed with synovial cells from 3 to 6 passages. SFB were used in proliferation (V) studies.

4.2 Synovial tissue explant cultures

Synovial tissue was obtained from knee arthroplasties of patients fulfilling the revised criteria of the American College of Rheumatology (Arnett et al. 1987). The study was approved by the ethical committee of surgery of the Helsinki University Central Hospital. Tissue explants were cultured in Dulbecco′s modified Eagle′s medium. Cultures of synovial tissue explants were used to study mast cell apoptosis (III), histamine release and cytokine production (III and IV), and TNF-α (III) and IL-1β immunohistochemistry (IV).

4.3 Activation of mast cells

4.3.1 Human mast cell line cells

HMC-1 cells were activated with a combination of PMA (50 ng/ml) and calcium ionophore A23187 (0.5 μM). This activation has been shown to produce an optimal cytokine release from HMC-1 cells (Lippert et al. 1996). The studied drug compounds were added 1 h prior to activation. Because activation reduces HMC-1 viability to some extent during the 24 h incubation, the cells were stained with trypan blue and cell numbers were counted after incubation.

4.3.2 Rat peritoneal mast cells

RPMC cells were activated with compound 48/80 (5 μg/ml), which induces histamine release from RPMC (Bloom and Haegermark 1965). The studied drug compounds were added 1 h prior to activation. 20 min after activation cells and supernatants were separated by centrifugation and frozen for later analysis.

4.3.3 Human mast cells obtained by culturing CD34+ stem cells

HuMCs were first incubated with human IgE to coat the FcεRI and the studied drug compounds were added 1 h before the activation. Thereafter HuMCs were activated by adding polyclonal anti-human anti-IgE. Culture media was collected 1 h later for histamine analysis.

4.3.4 Human synovial tissue mast cells

Human synovial tissue mast cells were activated with rabbit IgG antibodies against human IgE (150 μg/ml). A nonspecific rabbit IgG (150 μg/ml) was used as a control. A sample for histamine assay was taken after incubation for one hour and analysed for histamine content. The explants were incubated for 20-24 h, and then embedded in the O.C.T. compound-containing mould and frozen in liquid nitrogen. Culture media was collected and both explants and media were stored at -80 °C.

4.4 Drug compounds

Chemically modified tetracycline -1 (4-dedimethylamino tetracycline), CMT-3 (6-demethyl 6-deoxy 4 de-dimethylamino tetracycline), CMT-8 (6-α deoxy 5-hydroxy 4 de-dimethylamino tetracycline), CMT-308 (9-aminoCMT-3) were kindly provided by CollaGenex Pharmaceuticals (Newtown, PA, USA). Doxycycline was purhaced from Sigma (St.Louis, MO, USA). Imatinib mesylate was a kind gift from Novartis (Basel, Switzerland).

4.5 Measurements of mediators

4.5.1 ELISA

To determine histamine release, RPMC (I) or HuMC (I) or tissue explants (III, IV) were treated with the studied drugs and activated with specific substances (see 4.3). RPMC were incubated for 20 min and HuMC and tissue explant cultures were incubated for 1 h before taking samples for histamine analysis. Histamine levels in the culture media were measured with a colorimetric ELISA assay (IBL, Hamburg, Germany). In experiments where also intracellular histamine levels were measured (I), both cells and supernatants were subjected to three freeze thaw cycles to release the intracellular histamine from cell samples prior to ELISA-measurements.

To study cytokine secretion, HMC-1 cultures (I) or cultures of synovial tissue explants (III, IV) were incubated 20-24 hours after activation. Culture media were analysed by colorimetric ELISA-assays for TNF-α (I, III, IV), IL-8 (I), IL-1β (IV) IL-1Ra (IV) and VEGF (IV) levels. In experiments where also intracellular TNF-α levels were measured (I), both cells and supernatants were subjected to three freeze thaw cycles to release the intracellular TNF-α prior to ELISA measurements.

4.5.2 Messenger RNA

The level of TNF-α mRNA in HMC-1 cells (I) and the levels of TNF-α, IL-1β and VEGF mRNA in synovial tissue (IV) were determined by RT-PCR analysis. 5 x 10^6 HMC-1 cells were cultured with or without CMT-3 for 1 h and then activated. After 2 h culture, total RNA was isolated with RNeasy columns (I). Synovial mast cells were activated with anti-IgE and tissue samples were collected 24 h later. Approximately 30 mg synovial tissue was then homogenized and total RNA was isolated with RNeasy columns. RNAs were then subjected to reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR products were quantified with a GelDoc 2000 system (Bio-Rad).

4.6 Determination of cell proliferation

4.6.1 Cell numbers

HMC-1 were suspended into fresh supplemented Iscove's medium to density of 4 x 10^5 cells/ml. Cells were counted and cell viability was assessed by trypan blue staining on the fifth day after seeding (II, III).

mBMMC were cultured in IL-3 or rKL containing media in density of 4 x 10^5 cells/ml. The cells that were changed from IL-3 media to rKL media were cultured in rKL containing media two days before starting the experiment. Cells were counted and cell viability was assessed by trypan blue staining on the fifth day after seeding (II, III).

4.6.2 Measurement of [3H]thymidine incorporation

HMC-1 cells (1 x 10^5) were seeded on a 24-well plate and drugs and 0,5 µCi of [^{3}H]thymidine were added to the wells. Cells were harvested 24 hours later on glass microfiber filters. [^{3}H]thymidine incorporation into cellular DNA, i.e radioactivity of the samples, was measured by liquid scintillation counter (II, III).

4.6.3 BrdU assay

Synovial fibroblasts were trypsinized and 1 x 10^4 cells were seeded on 96-well plates in DMEM. After starving without growth factors for 48 h the cells were cultured in DMEM supplemented with 2 % FCS or 50 ng/ml PDGF. When anti-PDGF antibodies were used, it was added to culture media 2 h before using the media for cells. Imatinib was added into culture media and the plate was incubated in +37 °C. After 24 h incubation BrdU substrate was added to the cells and the cellular uptake of BrdU was measured colorimetrically 24 h later (V).

4.7 Detection of apoptosis

4.7.1 Cell death detection ELISAplus assay

HMC-1 or mBMMC were seeded into specific culture media (see 4.1) to density of 4 x 10^5 cells/ml and exposed to drugs for 24 h. Then the cell fractions were analyzed with the Cell death detection ELISAplus assay for internucleosomal degradation of genomic DNA typical for apoptosis (II, III). Campthotecin (5 µg/ml) induced cell apoptosis was used as a positive control.

4.7.2 TUNEL labelling

Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) with ApoTag® In Situ Apoptosis Detection kit. After 24 h culture with or without CMT-3 (II) or imatinib (III), 2 x 10^5 HuMCs (II, III) or samples of synovial tissue (III) were labelled with digoxigenin-conjugated dUTP and terminal deoxyribonucleotide transferase. The labelled DNA fragments were stained with anti-digoxigenin monoclonal antibody linked with fluorescein and viewed under fluorescence microscope.

4.8 Analysis of apoptotic mechanism

4.8.1 Measurement of caspase-3, -8 and -9 activity

The activities of caspase-3, -8 and -9 in HMC-1 cells (2 x 10^6) were measured after 6 h incubation with imatinib (III) or 20 h incubation with CMT-3 (II) by fluorometric (III) or colorimetric (II) activity assays for each specific caspase.

4.8.2 Detection of Bcl-2 and Bax

The expression of Bcl-2 protein and mRNA of Bcl-2 and Bax were determined in HMC-1 (II). The cells were cultured in the presence or absence of CMT-3 for 20 h. For the western blot analysis the cell pellet was lysed and the protein extracts were separated on a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membranes were incubated with specific antibodies against Bcl-2 and the primary antibodies were detected with horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG and an enhanced chemiluminecence detection system. The blots were quantitated with a Gel Doc 2000 system (Bio-Rad).

To determine the production of Bcl-2 and Bax mRNA in response to CMT-3 treatment, HMC-1 (5 x 10^6) cells were treated with CMT-3 for 20 h and thereafter total RNA was isolated using RNeasy columns. Bcl-2 and Bax mRNA expression was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Primers used in PCR are listed in Table 3.

4.9 Immunohistochemistry

4.9.1 Double-labelling immunohistochemistry

Double labelling immunohistochemistry was used to localize TNF-α (III) and IL-1β (IV) positive cells in RA synovial tissue. Mast cells were identified by staining for tryptase using mouse anti-human mast cell tryptase and Mouse Elite Kit or Alexa-fluor (594 or 488) goat anti-mouse antibodies. Macrophages were identified by staining for CD68. TNF-α was detected with polyclonal goat antibodies against

TNF-α and IL-1β were detected with polyclonal goat antibodies against IL-1β and Alexa-fluor (594) rabbit anti-goat antibodies. Specificity was controlled by negative IgG-control and by omitting the primary antibody.

4.9.2 Thy-1 and CD68

Immunohistochemistry was used to characterise the cultured synovial cells and to estimate the amount of contaminating macrophages. Cells (7,5 x 10^4) were cultured on coverslips for 48 h and fixed with paraformaldehyde (1%) and methanol. Non-specific binding was blocked with 1% milk (Thy-1 staining) or 3 % BSA buffer (CD68 staining) before staining the cells with monoclonal antibodies for fibroblast specific Thy-1 or macrophage specific CD68 antibodies. After overnight incubation at +4 °C with the primary antibodies, cells were incubated for 1 h with HRP-coupled rabbit-anti-mouse antibodies and stained with AEC. Finally, the cells were stained with haematoxylin. Specificity was controlled by negative IgG-control and by omitting the primary antibody.

4.10 Studies on PKC activity

We studied the effect of CMT-3 on PKC activity in intact HMC-1 cells and in vitro with rat brain PKC extract and human recombinant PKC α and δ isoforms (I).

To examine the effect of CMT-3 on the activation of mast cell PKC, we determined [^{3}H]phorbol-12,13-dibutyrate binding in intact HMC-1 cells. The method is based on the binding of phorbol esters only to the membrane-associated, activated PKC. HMC-1 cells were treated with CMT-3 for 5 min before PKC activation was stimulated with 1 μM of the A23187.

To assay the rat brain PKC activity, brain tissues from 3–4 rats were homogenized and eluted through a chromatography column containing DEAE Sephacel (Pharmacia, Sweden). The human recombinant PKC α and δ isoforms were cloned and expressed in baculovirus-infected Sf9 insect cells as described by Tammela et al. (2004).

The kinase activity of PKC was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into PKC-specific substrate peptide (FKKSFKL-NH_2, Chakravarthy et al. 1991).

4.11 Statistical analysis

The results are expressed as mean ± SEM. One-way ANOVA/Tuckey's test (I, II, V) and ANOVA/Dunn′s test (III) and paired t-test (IV) were used to assess significance between controls and treatments. $P < 0.05$ was considered to be significant.

5.
RESULTS

In this chapter the results of this thesis are summarised. The majority of figures is presented in the respective original publications I-V.

5.1 CMT-3 inhibits histamine release and cytokine production in cultured mast cells (I)

The objective of this study was to determine the effects of CMT-3 on histamine release and on the production of two important cytokines, the proinflammatory TNF-α and the chemotactic IL-8 in cultured mast cells.

CMT-3 inhibited the activation-induced histamine release from cultured rat and human mast cells. CMT-3 induced a significant inhibition of compound 48/80-induced histamine release from RPMC (I, figure 1A). Treatment of RPMC with 25 μM CMT-3 reduced the degranulation percent from 71.7 ± 4.2 % to 28.7 ± 3.4 % ($p = 0.0013$). In HuMCs activated via IgE-receptors, there was a non-significant tendency of inhibition by CMT-3 (I, figure 1B).

CMT-3 inhibited PMA-ionophore A23187 -induced TNF-α and IL-8 secretion from HMC-1 cells. In the presence of 25 μM CMT-3, the secretion of TNF-α was reduced by 86.5 ± 4.1 % ($p = 0.00023$) in comparison to activated control (I, figure 2A). Similarly, treatment of HMC-1 with 25 μM CMT-3 reduced the IL-8 secretion by 90.3 ± 1.1 % ($p = 0.00014$) in comparison to activated control (I, figure 2B).

The quantities of intracellular and secreted TNF-α in resting and activated HMC-1 cells in the presence of CMT-3 were measured simultaneously and the levels of TNF-α mRNA were analysed to determine whether CMT-3 inhibits the production and/or the release of TNF-α from the HMC-1 cells. In the presence of 25 μM CMT-3, a significant decrease in both intracellular and secreted TNF-α was evident when compared to the activated control cells (I, figure 3). Furthermore, 25 μM CMT-3 attenuated the activation induced increase in TNF-α mRNA expression, although the change was not statistically significant (I, figure 4). These data strongly indicates that CMT-3 inhibits the production and not just the release of TNF-α in HMC-1 cells.

In comparison to doxycycline, CMT-3 was a clearly more potent inhibitor of histamine release and cytokine production in mast cells. Doxycycline did not have an appreciable effect on the histamine release by activated RPMC in the concentration range studied (I, figure 1A). Doxycycline did inhibit TNF-α and IL-8 secretion (I, figures 2A and 2B), but less effectively than did equimolar concentrations of CMT-3.

5.2 The activity of PKC is inhibited by CMT-3 (I)

This study showed, for the first time, that CMT-3 inhibits the activity of PKC. The effect of CMT-3 on PKC activity was investigated in three different model systems: in intact HMC-1 cells, in rat brain homogenate and using human recombinant PKC isoforms in Sf9 cells lysates.

First, the effect of CMT-3 on [^{3}H]-phorbol 12,13-dibutyrate-binding in intact HMC-1 cells stimulated with Ca-ionophore A-23187 was investigated. CMT-3 decreased the incorporation of [^{3}H]-phorbol 12,13-dibutyrate in a dose-dependent manner (I, figure 5A). This result suggested that CMT-3 might act as a PKC inhibitor. Although, recent studies have shown that cells may express also other phorbol ester receptors than PKC (Kazanietz 2002), which might influence the results.

The next step was to determine, whether CMT-3 could inhibit the activity of isolated PKC enzymes *in vitro*. CMT-3 inhibited PKC activity in rat brain homogenate, known to be rich in PKC, in a dose-dependent manner with an IC_{50} value of about 30 μM (I, figure 5B). CMT-3 was a highly effective inhibitor in comparison to doxycycline, which did not affect the activity of PKC at the concentrations studied. This is consistent with the finding by Webster et al. (1994), that doxycycline inhibits PKC activity with clearly higher, pharmacologically irrelevant, drug concentrations.

The PKC family comprises of at least 12 different isoforms. Since PKC alpha and delta are critical PKC isoforms in mast cell function, the effect of CMT-3 on these isozymes was investigated more closely by using recombinant human PKC alpha and delta. CMT-3 inhibited markedly the activities of both PKC alpha and PKC delta (I, figure 5C).

5.3 CMTs inhibit proliferation and induce apoptosis of cultured mast cells (II)

CMTs are known to induce apoptosis in several malignant cell lines. In this study, the ability of four CMTs to inhibit proliferation and induce apoptosis of cultured mast cells was investigated.

All studied CMTs reduced significantly the viability of HMC-1 cells (II, figure 1A). In the presence of 25 μM of CMT-3, the number of viable cells decreased profoundly during five days of culture (to 12.3 ± 8.0 % of untreated control). CMT-8 also decreased the viability of HMC-1 cells (to 17.6 ± 0.9 % of untreated control) to a similar extent as CMT-3. Moreover, CMT-1 and CMT-308, had a clear effect on the viability of HMC-1, however, they were less potent than CMT-3 and CMT-8. The antiproliferative effect of CMT-3 and CMT-8 was confirmed by showing that CMT-3 also decreased the [^{3}H]thymidine incorporation (II, figure 1C).

The viability of mBMMC can be sustained either by IL-3 or c-kit ligand. To assess the dependency of the antiproliferative effect of CMT-3 on growth factor, mBMMC were cultured in the presence of IL-3 or c-kit-ligand and the effects of CMT-3, CMT-8 and doxycycline were assessed. In the presence of CMT-3, the viability of mBMMC was significantly reduced irrespective whether they were cultured in the presence of IL-3 or c-kit-ligand (II, figure 2). After five days in culture, no living cells could be observed in the presence of 10 µM or 25 µM of CMT-3. Similar results were observed for CMT-8.

Next we studied whether the observed inhibition of viability was due to apoptosis. CMTs induced a significant and dose-dependent increase in the amount of apoptotic nucleosomes both in HMC-1 cells (II, figure 3A) and in mBMMC (II, figure 3B). In accordance with the proliferation experiments, the effect of CMT-8 was in the same range while doxycycline was rather ineffective. Incubation of HuMCs for 24 h with CMT-3 resulted in a higher number of TUNEL positive HuMCs indicating increased rate of apoptosis (II, figure 4). The finding was important as it confirms that also non-malignant human mast cells are prone to CMT-3 induced apoptosis.

When studying the mechanism of apoptosis in HMC-1 cells in response to treatment with CMT-3, we observed increases in caspase-3 and caspase-9 activities (II, figure 5). Furthermore, the protein levels of anti-apoptotic Bcl-2 were decreased (to 68.4 ± 16.7% in comparison to control cells (II figure 6A) in CMT-3 treated HMC-1 cells. Simultaneously, mRNA levels of proapoptotic Bax were increased (to 145.5 ± 27.0 % in comparison to control cells, II, figure 6C), suggesting increased rate of Bax synthesis in HMC-1 cells following CMT-3 treatment. However, also mRNA levels of Bcl-2 were increased (to 131.0 ± 18.2 % in comparison to control cells, II, figure 6B), most likely due to a compensatory increase of mRNA expression induced by the reduced Bcl-2 protein level.

This study showed that CMTs have an antiproliferative and apoptosis-inducing effect on both malignant and non-malignant mouse and human mast cells. Of the studied four chemically modified tetracyclines (CMT-1, CMT–3, CMT-8 and CMT-308), CMT-3 was the most potent compound. Doxycycline was relatively ineffective in inhibiting proliferation and inducing apoptosis in mast cells.

5.4 Imatinib induces apoptosis of cultured and synovial mast cells (III)

Among a few tyrosine kinases, imatinib inhibits c-kit receptor, which mediates the effects of SCF, i.e. the most important growth factor for human mast cell viability. The purpose of this study was to elucidate whether blocking c-kit with imatinib induces apoptosis in human mast cells.

Imatinib exerted a highly antiproliferative effect on HMC-1 cells. A profound decrease in cell number was observed after 5 days of culture already in the presence of 0.1 μM of imatinib and, in the presence of 1 and 10 μM of imatinib, only a few living cells were detected after 5 days of culture (III, figure 1A).

To verify that the effects of imatinib were mediated via c-kit inhibition, we studied mBMMCs, which were cultured in the presence of SCF or IL-3. When the mBMMCs were cultured in the presence of SCF and imatinib, there was a strong reduction in the cell number at 1 μM and higher imatinib concentrations (III, figure 1B). In contrast, when mBMMCs were cultured in the presence of IL-3, imatinib did not significantly reduce the cell number, suggesting that imatinib specifically affected the c-kit-dependent survival pathway.

The observed reduction in HMC-1 cell number was caused by apoptosis as judged by typical apoptotic morphology, increased number of apoptotic nucleosomes (III, figure 1C) and activation of caspase-8 and -9 (III, figure 1D). Additional methods to detect apoptosis, including annexin binding and TUNEL staining, confirmed the finding. Thus, inhibition of c-kit signalling in HMC-1 cells activates a metabolic cascade, which induces cell death by apoptosis.

In HuMCs, cultured in the presence of SCF and an increasing concentration of imatinib, a significant increase in the number of apoptotic nucleosomes was observed (III, figure 2A). The induction of human mast cell apoptosis by imatinib was further verified by studying nuclear fragmentation (III, insert in figure 2A), TUNEL staining (III, figure 2B), and annexin binding (data not shown). Interestingly, in the presence of another supporting cytokine (IL-6), in addition to SCF, human mast cells were more resistant to apoptosis (III, figure 2A).

In inflamed synovial tissue, there are also other potential cytokine and growth factor pathways, in addition to the c-kit pathway, which can support the viability of tissue mast cells. Therefore it was important to study the effect of imatinib on the viability of tissue mast cells. Culturing rheumatoid synovial tissue explants with imatinib induced apoptosis in synovial tissue during 24 h incubation (III, figure 5). Most of the apoptotic cells were mast cells as assessed by double-staining with TUNEL and anti-tryptase, and the majority of mast cells in rheumatoid synovial tissue were rendered apoptotic during the incubation with imatinib.

In summary, this study showed that by inhibiting c-kit, imatinib exerts a proapoptotic effect both on cultured mast cells and, importantly, on mast cells resident in RA synovial tissue.

5.5 Activation of synovial mast cells results in production of cytokines (III and IV)

Activated mast cells produce and secrete a remarkable array of proinflammatory mediators. The objective of this study was to determine the effects of synovial mast cell activation on the production of TNF-α, IL-1β, IL-1Ra and VEGF by synovial tissue.

Histamine release was determined to verify the activation of synovial mast cells (IV, figure 1). Anti-IgE induced a significant histamine release from synovial tissue explants derived from 12 patients (0.84 ± 0.91 vs. 2.10 ± 1.07 pg/mg wet tissue, p = 0.0065) during 1 h culture. Notably, the variation in the basal histamine levels in the control tissue cultures was high. However, in each case, except one, addition of anti-IgE resulted in histamine release from the explants.

Selective activation of synovial tissue mast cells by anti-IgE induced a highly significant increase in the secretion of TNF-α in comparison to untreated control (4.56 ± 3.18 vs. 0.49 ± 0.88 pg/mg wet tissue, p = 0.00037) from explants to culture media during the 24 h culture (IV, figure 2A). These experiments were done with synovial tissue from 13 RA patients. The main fraction of the secreted TNF-α by synovial tissue was shown to be newly synthesized, as no detectable amounts of TNF-α were found in tissue culture media samples taken 1 h after mast cell activation. The data from TNF-α mRNA studies supported this conclusion, as it indicated an increased rate of TNF-α synthesis as a result from mast cell activation (IV, figure 2C). The immunohistochemical studies revealed that RA synovial tissue contains abundant number of TNF-α positive mast cells (III, figure 3).

Activation of mast cells in synovial tissue explants induced a significant increase in the secretion of IL-1β in comparison to untreated control (2.55 ± 1.98 vs. 0.058 ± 0.032 pg/mg wet tissue, p = 0.013) during the 24 h incubation (IV, figure 3A). Synovial tissue from 11 RA patients was used for these experiments. The increase in IL-1β secretion was most likely due to an increased synthesis rate, as anti-IgE induced an elevation in IL-1β mRNA level in synovial tissue (IV, figure 3C). Immunohistochemical studies showed that when mast cells were activated, a clear IL-1β positivity appeared in synovial tissue (IV, figure 4). To study in more detail which cell types expressed IL-1β, double labelling immunohistochemistry was performed. Mainly synovial macrophages were found to be IL-1β positive in tissue explants after mast cell activation by anti-IgE. These results indicate that activation of mast cells in synovial tissue induces IL-1β expression in synovial macrophages.

The untreated control explants of rheumatoid synovial tissue secreted a considerable amount of IL-1Ra into tissue culture media (24.0 ± 19.7 pg/mg wet tissue, IV, figure 5A). Addition of non-specific IgG elevated the secretion of IL-1Ra to culture media (70.3 ± 20.8 pg/mg wet tissue, p = 0.0006). Yet, the increase in the secretion of IL-1Ra was clearly more pronounced in anti-IgE-stimulated samples (132.3 ± 17.3 pg/mg wet tissue, p = 0.0000), revealing the involvement of mast-cell-

dependent component in the secretion of IL-1Ra following addition of anti-IgE. Noteworthy, the relative increase of IL-1Ra was clearly smaller than the relative increase of IL-1β. These experiments were performed with tissue explants from 7 RA patients (IV, figure 5B).

The level of VEGF in tissue culture media 24 h after synovial mast cell activation was clearly decreased in comparison to untreated control (5.62 ± 2.3 vs. 8.20 ± 3.7 pg/mg wet tissue, p = 0.035, IV, figure 6A). The unexpected decline in VEGF was observed consistently in all studied 7 patient samples (IV, figure 6B). On the contrary, the simultaneous VEGF mRNA expression seemed elevated, although the change was not statistically significant.

In summary, these experiments show that the production of TNF-α and IL-1β by RA synovial tissue explants was significantly increased as a result of mast cell activation.

5.6 Imatinib inhibits anti-IgE-induced TNF-α secretion in rheumatoid synovial tissue (III)

In the presence of imatinib, the secretion of TNF-α by RA synovial tissue explants induced by IgE-dependent activation of mast cells was significantly decreased (III, figure 4A). However, imatinib did not affect acute histamine release by the RA synovial tissue (III, figure 4B). The finding was consistent with the observation, that imatinib did not have an effect on histamine release from cultured mast cells. As imatinib was shown to induce apoptosis in synovial mast cells, the observed decrease in TNF-α secretion might be due to a decline in the number of viable mast cells in tissue explants treated with imatinib.

5.7 CMT-3 inhibits anti-IgE-induced TNF-α and IL-β secretion in rheumatoid synovial tissue (unpublished data)

In order to assess the potential benefits of CMT-3 in treatment of RA, the effects of CMT-3 on the secretion of the key cytokines, TNF-α and IL-1β, by RA synovial tissue were determined.

Anti-IgE induced histamine release from synovial tissue explants was not affected by pretreatment with CMT-3 (Figure 7). The slight decrease in histamine release by 25 μM CMT-3 was insignificant. This result is consistent with the previous finding, that CMT-3 was rather ineffective in inhibiting histamine release from HuMC (I).

As shown in Figure 8, CMT-3 decreased significantly the mast cell activation induced secretion of TNF-α from RA synovial tissue in comparison to untreated tissue explants (2.45 ± 2.14 vs. 5.04 ± 3.23 pg/mg wet tissue, p = 0.001). This result

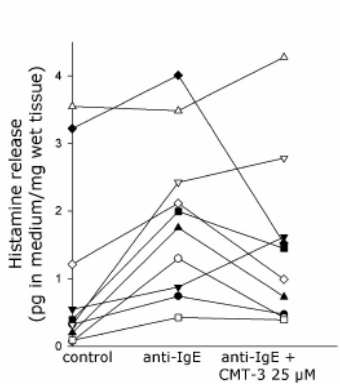

Figure 7. The effect of CMT-3 on the histamine release by synovial tissue explants stimulated with anti-IgE.
The explants were incubated with the indicated substances. CMT-3 was added 1 h before activation of synovial mast cells with anti-IgE and tissue culture media was collected 1 h later. Histamine levels in culture media were analyzed with ELISA assay. Values of 10 individual patients are shown.

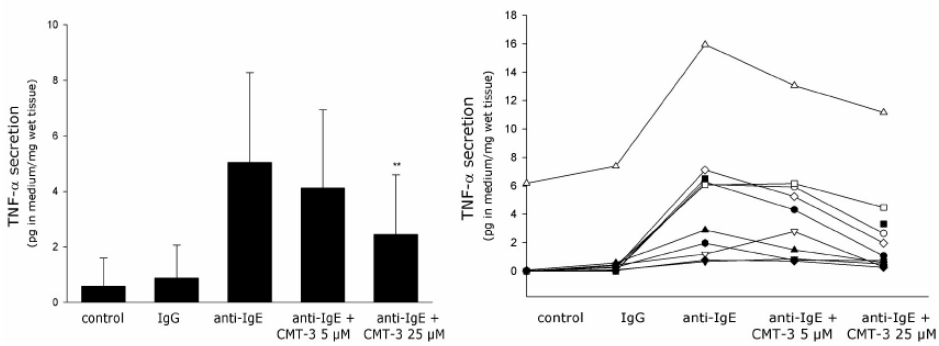

Figure 8. IgE-dependent mast cell activation induces TNF-α secretion from RA synovial tissue.
The explants were incubated with the indicated substances. CMT-3 was added 1 h before adding anti-immunoglobulins and culture media was collected 24 h later. TNF-α levels in culture media were analyzed with ELISA assay. A) Means ± SEM and B) individual values of 11 different patient samples are shown. ** =p < 0.01

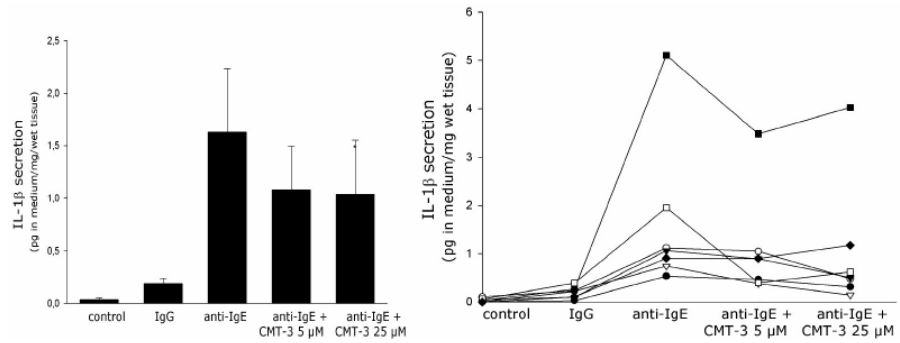

Figure 9. IgE-dependent mast cell activation induces IL-1β secretion from RA synovial tissue.
The explants were incubated with the indicated substances. CMT-3 was added 1 h before adding anti-immunoglobulins and culture media was collected 24 h later. IL-1Ra levels in culture media were analyzed with ELISA assay. A) Means ± SEM and B) individual values of 7 different patient samples are shown. * = p < 0.05

is in accordance with observations in HMC-1 cells, which showed that CMT-3 effectively inhibits the production of TNF-α in activated mast cells (I).

Figure 9 shows that CMT-3 decreased significantly the mast cell activation induced secretion of IL-1β from RA synovial tissue in comparison to untreated control explants (1.04 ± 0.51 vs. 1.63 ± 0.60 pg/mg wet tissue, p= 0.025).

These experiments show that 25 μM CMT-3 is a potent inhibitor of cytokine secretion induced by activation of mast cells in RA synovial tissue. If the observed inhibitory effect could be accomplished in vivo, CMT-3 might possess antirheumatic properties.

5.8 Imatinib mesylate inhibits proliferation of rheumatoid synovial fibroblasts (V)

The exaggerated proliferation of synovial fibroblasts (SFB) is a central feature in the development of pannus tissue in RA. As PDGF is an important mitogen for SFB and imatinib inhibits the PDGF receptor tyrosine kinase, this study was designed to determine the effect of imatinib on SFB proliferation.

PDGF stimulated cultured SFB to proliferate and the addition of 1 μM imatinib completely inhibited the PDGF-dependent increase in uptake of BrdU (V, figure 2A). The inhibition of PDGF-stimulated proliferation of SFB by imatinib was dose-dependent in the 0.01–10 μM concentration range. Similar extent of inhibition of PDGF-stimulated proliferation was observed when PDGF was neutralized with anti-PDGF-antibodies, as compared to inhibition with 1μM imatinib.

FCS also stimulated the proliferation of SFB. In the presence of imatinib, the FCS-stimulated uptake of BrdU was significantly, but not completely reduced (V, figure 2B). Neutralizing the PDGF in serum by anti-PDGF antibodies also reduced the uptake of BrdU by SFB. The inhibition of BrdU uptake by 1 μM imatinib was comparable to the inhibition observed with anti-PDGF antibodies Furthermore, if imatinib was added to media in which PDGF had been neutralized with anti-PDGF antibodies, no further inhibition of BrdU uptake was observed. These results suggest that imatinib specifically prevents the action of PDGF in serum, leaving signalling pathways stimulated by other growth factors intact.

6.
DISCUSSION

In this chapter, the results of the present study are discussed as an entity. A more detailed discussion is presented in the original publications I-V.

6.1 Mast cell in synovial inflammation

Given the increased numbers of mast cells within the inflamed joint and the exceptionally wide range of potent mediators secreted by activated mast cells, they are likely to contribute the initiation and upholding of chronic inflammation. We used synovial tissue from RA patients to study the consequences of mast cell activation in the context of synovitis associated with RA.

The rheumatoid synovium, which is relatively easily accessible, is one of the most extensively analysed tissue sites of a local immune and inflammatory reaction. For surgical reasons, the rheumatoid tissues examined were derived almost exclusively from middle or late stage disease. As the synovial tissue was used for the experiments within hours after operation, the prevailing treatments of the patient might have affected the function of mast cells in the synovial explants. Thus, disease duration, treatments and other patient-related data were collected and reported (IV, Table 1).

Mast cell activation may be induced in vivo by several mechanisms. In this study, polyclonal anti-human-anti-IgE was used as a mast cell secretagogue as it selectively activates mast cells via their FcεRI receptors. It is unlikely that IgE-mediated mast cell activation is involved in RA, although IgE rheumatoid factors and immune complexes with IgE have been described in small subsets of RA patients (De Clerck et al. 1989, Gruber et al. 1988b). In RA, the synovial mast cells could be expected to be activated by complement fragments such as C5a, and by immune complexes comprised of IgG (Nigrovic and Lee 2005, Benoist and Mathis 2002). Receptors for complement fragment C5a are expressed on mast cells in synovium of RA patients (Kiener et al. 1998). Yet, in RA synovial tissue a significant proportion of mast cells are activated. Therefore, it is of great importance to study the consequences of mast cell activation in synovial tissue, irrespective of the means of activation. Activation with anti-IgE provides a reliable means to selectively activate mast cells and to study the consequences of their activation in synovial tissue under experimental conditions. In future studies, research on the factors which activate human synovial mast cells and the consequences of mast cell activation by C5a or immune complexes

on cytokine production would be interesting approaches to further clarify the role of mast cell activation in RA.

Selective activation of mast cells induced a significant increase in the production of TNF-α and IL-1β in rheumatoid synovial tissue explant cultures during the 24 h culture. We showed that synovial mast cell activation induced IL-1β expression in synovial macrophages. One explanatory mechanism behind this observation could be that mast-cell-derived TNF-α stimulates synovial macrophages to produce IL-1β. Synovial macrophages were identified with immunostaining for CD68, which is a widely used marker for macrophages. It should be noted that also human mast cells have been shown to express CD68 (Nilsson et al. 1996, Horny et al. 1990). If these intriquing observations can be verified it could mean that some of the tissue cytokine expression attributed in previous studies to macrophages could in fact be derived from mast cells. Therefore it would be of importance in the future to study whether also synovial mast cells express CD68.

In accordance to the present results, Woolley and Tetlow (2000) have shown previously by immunohistochemistry that in rheumatoid synovial tissue cytokine expression can be observed in the vicinity of mast cells. However, in the cited study the authors report that mast cell activation decreased the secretion of TNF-α and IL-1β in synovial tissue explants. The results of our study contradict these earlier findings. We observed a significant and systematic increase in TNF-α and IL-1β levels in 13 and 11 patient samples, respectively. Furthermore, the appearance of enhanced expression of cytokine mRNA and the appearance of IL-1β protein observed by immunohistochemistry as a result of mast cell activation are shown. These findings strongly support the concept that mast cell activation indeed results in cytokine production in synovial tissue.

We also observed a significant increase in the secretion of anti-inflammatory IL-1Ra into the tissue culture media. However, the elevation in IL-1Ra was clearly less pronounced that that of IL-1β, i.e. the ratio of IL-1β/IL-1Ra was much lower in the culture media of control explant (1/414) than in anti-IgE-treated explant cultures (1/52). Less than 5% of IL-1 receptors need to be engaged by IL-1 in order to induce a biological response, and it is estimated that a 10- to 100-fold excess of IL-1Ra is necessary to achieve a 50% inhibition of the IL-1 response (Arend et al. 1990). Thus activation of mast cells in synovial tissue cultures increased the IL-1β/IL-1Ra ratio by 8-fold, so favouring a proinflammatory effect.

Several types of cells have been shown to trigger synovial inflammation. The autoimmune background of RA emphasizes the role of T cells in the ethiology of the disease. However, also mast cells are present in synovium in abundance. Their capability to secrete an exceptionally wide array of cytokines makes them a potential player in the pathophysiology of RA. The essential role for mast cells in the development of autoantibody-induced arthritis in mice has been recently described by Lee et al. (2002). Previous studies on the human synovial mast cell activation are limited. Woolley with his colleagues has done fundamental research on the role

of mast cells and on the consequences of mast cell activation in RA synovial tissue (Bromley et al. 1984, Bromley and Woolley 1984, Tetlow and Woolley 1995a and 1995b, Tetlow et al. 1998, Woolley and Tetlow 2000).

The present findings extend the earlier knowledge about the role of mast cells in RA. We found that mast cell activation results in significant production of TNF-α and IL-1β. Several lines of investigation, including experimental and clinical studies, have strongly emphasised the importance of these cytokines in the pathogenesis of RA (Firestein 2003, Feldmann and Maini 2001, Kay and Calabrese 2004). When summarised, our results indicate that mast cells can significantly contribute to the pathogenic mechanisms in the synovium that result in erosive arthritis. These data also raise the possibility that targeting mast cells in synovial tissue may provide a novel strategy for therapeutic intervention in RA.

6.2 CMT-3 and imatinib as inhibitors of mast cell function

This study demonstrates that CMT-3 is an effective inhibitor of cytokine production by mast cells. The effect of CMT-3 is dose-dependent and significant responses in mast cells are evoked with 10–25 μM concentrations. The results from analysis of intracellular TNF-α levels and TNF-α mRNA expression strongly suggest that CMT-3 did not just prevent TNF-α secretion but inhibited the synthesis of TNF-α. This is likely due to inhibition of mast cell activation. Secondly, this study shows that CMTs exert an antiproliferative and proapoptotic effect on mast cells. The present results on HMC-1 cells are in accordance with several recent studies, which have described that CMTs have antiproliferative activity in several cancer cell lines (D'Agostino et al. 2003, Lokeshwar et al. 2002, Tolomeo et al. 2001, Meng et al. 2000, Seftor et al. 1998). However, the present study is the first to show that CMT-3 induces apoptosis in non-malignant human immune system cells.

In the first study, which described inhibition of cytokine production in activated mast cells by CMT-3, we did not notice significant reduction in cell numbers during the 24 h culture period. Nevertheless, according to the results from the second study, mast cells are susceptible to CMT-induced apoptosis. Activation of mast cells has been shown to protect them from apoptosis by inducing the expression of the antiapoptotic molecules (Xiang et al. 2001, Möller et al. 2003). This could explain the contrasting results of our two studies concerning the cell numbers. This would also mean, that mast cells which are most likely in activated state in inflamed tissues, would be less prone to CMT-induced apoptosis. Additionally, in the second study the decrease in cell numbers was observed after 5 days of culturing with CMT-3, while the cytokine production was measured after 24 h of culturing with CMT-3. Related to the study II, we observed also that the cell numbers were not significantly decreased after 24 h (unpublished data), although the signs of apoptosis, such as apoptotic nucleosomes and caspase activity, were detectable. Therefore, on the basis

of these studies, the possibility remains that at least part of the observed inhibition of cytokine production by CMT-3 is caused by the induction of mast cell apoptosis.

So far there have been only limited number of means to selectively inhibit mast cell functions. SCF critically regulates the migration and survival of mast cell precursors, promotes the proliferation of both immature and mature mast cells, enhances mast cell maturation, directly induces secretion of mast cell mediators, and regulates the extent of mediator release in mast cells activated by IgE-dependent mechanisms (Galli et al. 1993). With this background, it was rather predictable that imatinib, given its capacity to inhibit c-kit, the receptor of SCF, would have profound effects on mast cell function. This study showed that imatinib indeed is an effective inhibitor of mast cell viability within concentrations that are pharmacologically relevant (0.1–1 μM). Imatinib now provides a new interesting tool for the research of mast cell biology and to study the role of mast cells in different physiological and pathological conditions. The selectivity of imatinib towards only a few tyrosine kinases, in this case c-kit, makes it an attractive means to study mast cells. The observed proapoptotic effect of imatinib in rheumatoid synovium is in all likelihood relatively mast-cell specific, as in RA synovium c-kit is expressed exclusively by mast cells (Ceponis et al. 1998). As imatinib is already approved for use in humans and the safety profile of the drug is known (Savage and Antman 2002), the applications of basic research findings to clinical practise might be facilitated.

In these experiments, several mast cell models were used. HMC-1 is a malignant human mast cell line. The use of HMC-1 cells provides an easily accessible model of proliferating mast cells that have also the capability to secrete cytokines The lack of functional FcεRI-receptors is the major limitation of HMC-1 model. Human CD34+ stem cell derived mast cells (HuMCs) are at the moment the best *in vitro* model for human mast cells. These cells can be activated via their FcεRI-receptors and their phenotype is close to human MCTC-type mast cells. The time-consuming and expensive maturation process of CD34+ stem cells to mast cells limits the use of this model. In the present work, rodent mast cells were used as additional mast cell models in early screening of drug effects. RPMC is the most extensively studied mast cell model in the context of histamine release. mBMMC are primary mast cells that proliferate. mBMMC provides a means to study the effect of different growth factors on the proliferation.

In conclusion, both CMT-3 and imatinib were found to induce apoptosis in mast cells. Their mechanism of action differs most likely. Of these two compounds, imatinib probably provides relative selectivity against mast cells, while CMT-3 is likely to affect other cell types as well as mast cells. However, both compounds effectively inhibit mast cell functions and thus CMT-3 and especially imatinib could be used as novel drugs to inhibit mast cells.

6.3 PKC inhibition as a target for CMT-3

Protein kinase C comprises a family of enzymes implicated in cellular differentiation, growth control and tumour promotion. In this study we show, for the first time, that CMT-3 inhibits PKC activity. Of the several PKC isoenzymes, at least PKC alfa and PKC delta are inhibited by CMT-3 is evident. Considering the central role of PKC in numerous cellular processes, the observed capacity to inhibit PKC might open new perspectives for the clinical use of CMT-3.

The observed inhibitory effects of CMT-3 on mast cell function could be explained, at least partially, by the inhibition of PKC. PKC is expressed in different types of mast cells and it is required for both early and delayed responses (Nechushtan and Razin 2001). Of the different PKC isoforms, at least alpha, beta, delta, epsilon and theta are expressed in a rat mast cell line (Nechushtan and Razin 2001). In addition to PKC alpha and PKC delta, also PKC beta is implicated in the mast cell activation and it would have been rational to determine the effect of CMT-3 on this isoform as well. Unfortunately, PKC beta isoform was not available for this study. The pathways activated following crosslinking of FcεRI have been extensively studied and it is clear that PKC is involved in the cascade (Tsaczyk and Gilfillan 2001, Turner and Kinet 1999). Based on this, PKC is acknowledged as one attractive target for interfering the mast cell activation. Consistent with this, selective PKC inhibitors have been shown to block FcεRI-dependent mediator release of human mast cells (Noll et al. 1997, Amon et al. 1996, Kimata et al. 1999 and 2000). However, the precise targets for the PKC signals within the secretory machinery in mast cells are not known.

Whether the mechanism underlying the ability of CMT-3 to induce apoptosis in mast cells, is PKC inhibition or not, remains unclear. The previous findings on the role of PKC in mast cell apoptosis are somewhat contradictory. An enhanced proliferation rate in a rat mast cell line overexpressing PKC has been described (Chang et al. 1997), although PKC has been shown to play an inhibitory role in IL-3-mediated mouse mast cell proliferation (Chaikin et al. 1990, Baranes and Razin 1991).

PKC is central in signalling pathways involved in adaptive immune responses in mammals. PKC is known to regulate B cell activation and survival (Saijo et al. 2003) and PKC activation induces T cell proliferation and activation (Berry and Nishizuka 1990, Isakov and Altman 2002, Wilkinson and Nixon 1998). Considering the central role of T and B cells in pathoimmunological processes, PKC might offer a potential target to control autoimmune diseases. There is evidence from animal models that selective inhibitors of protein kinase C are useful in the treatment of adjuvant-induced arthritis (Birchall et al. 1994, Zhou et al. 1999). Given the autoimmune background of RA, CMT-3, besides inhibiting the cytokine production induced by mast cell activation in rheumatoid synovium, could be beneficial in treatment of RA as it could reduce the T and B cell driven processes by inhibiting PKC.

In addition to RA and other diseases associated with inappropriate mast cell activation, PKC inhibition has several other clinical implications, especially in oncology (Hofmann 2004, Lahn et al. 2004, Poole et al. 2004). The ability of CMT-3 to inhibit PKC activity might be a contributing factor in the recognized anticancer activity of CMT-3.

6.4 Prospective antirheumatic effect of CMT-3

Tetracyclines have been studied in the context of RA because of their anti-inflammatory properties and particularly because of their capability to inhibit MMPs (Greenwald 1994). A meta-analysis of the clinical trials on the use of tetracyclines in RA found that tetracyclines, in particular minocycline, are associated with a clinically significant improvement in disease activity in RA (Stone et al. 2003). However, contrasting results have also been obtained (van den Laan et al. 2001). Nevertheless, the studies share the conclusion that tetracyclines appear not to affect the radiological progression of the disease. In contrast, CMT-3 has been reported to have beneficial effect on the bone mass and bone integrity in arthritic rats (Zernicke et al. 1997).

Inhibition of structural damage is a primary therapeutic aim in RA. MMPs form a family of enzymes with a major a role in cartilage destruction associated with RA. Results from clinical trials with MMP inhibitors in RA have been unequivocal, with some studies being terminated because of lack of efficacy or safety concerns (Keystone 2004). Nevertheless, this approach has been considered promising (Martel-Pellertier et al. 2001, Elliot and Cawston 2001). Several studies have confirmed that CMT-3 is a potent inhibitor of several MMPs, with specificity towards the MMP-2, MMP-9, and MMP-14 isozymes (Acharya et al. 2004). All of these have been found to be overexpressed in RA synovium (Konttinen et al. 1999). In addition to inhibiting MMP activity, CMT-3 has been shown to induce apoptosis in human osteoclasts, which might contribute to its antiresorptive action in bone (Holmes et al. 2004). Moreover, CMT-3 was found to inhibit SFB invasion in an experimental animal model (Seftor et al. 1998). Taken together, preventing tissue destruction could be one dimension in the potential antirheumatic effect of CMT-3 (Sorsa et al. 1998).

The present study showed that CMT-3 inhibits effectively cytokine production in activated mast cells and in human synovial tissue stimulated with selective mast cell secretagogue. The inhibition of TNF-α and IL-1β production in synovial tissue might be a remarkable finding considering the antirheumatic potential of CMT-3. The previous observation, that CMT-3 effectively blocks PGE_2 production in chondrocytes and macrophages (Patel et al. 1999), strengthens the concept of CMT-3 as an anti-inflammatory agent in RA. Besides joint destruction and chronic inflammation, one prominent feature of RA is angiogenesis. CMT-3 has been shown to inhibit angiogenesis in vitro (Fife et al. 2000). This character of CMT-3

is believed to be important considering the anticancer activity, but it could also be beneficial in the treatment of RA.

Laroux et al. (1999) studied the effect of CMT-3 in a model of chronic polyarthritis in mice in which the arthritis was induced with monoclonal antibodies. They found that CMT-3 could possess beneficial activity in arthritis. In another study, Zernicke et al. (1997) detected positive effects by CMT-3 and CMT-8 on the amount of bone and the biomechanical properties of femoral neck in rat adjuvant arthritis. However, Zernicke et al. (1997) did not observe any anti-inflammatory effects in this arthritis model following CMT-3 treatment.

In summary, *in vitro* CMT-3 exerts several interesting properties, such as inhibition of MMPs, inhibition of cytokine and prostaglandin production, inhibition of osteoclast activity and inhibition of angiogenesis, which suggest efficacy in the treatment of RA. Based on the results of the present study and previous publications, further studies are warranted to evaluate the true potential of CMT-3 as an antirheumatic agent.

6.5 Imatinib as a novel antirheumatic drug

At present imatinib is approved for treatment of chronic myeloid leukemia and gastrointestinal stromal tumours. The unique mechanism of action and the relatively high selectivity of imatinib have also evoked interests concerning many other diseases, including RA. GIST-patient suffering also from RA was treated with imatinib at Helsinki University Central Hospital and an improvement in RA symptoms was observed during the treatment. This observation gave rationale to a 12 weeks pilot clinical study, which demonstrated a clinical improvement in three RA patients with active disease that had been resistant to earlier therapies (Eklund and Joensuu 2003). A case report, published by Miyachi et al. (2003), describes a similar finding. A single patient suffering from severe RA has been treated with imatinib for nearly 3 years at Helsinki University Central Hospital. The activity of RA was very low during the entire treatment period (unpublished data). These findings, although based on a very small patient population, suggest that imatinib might have significant antirheumatic activity.

Mast cells express c-kit (Tsai et al. 1991, Kirshenbaum et al. 1992) and synovial fibroblasts express PDFGR (Butler et al. 1989, Heldin and Westermark 1999), both of which are inhibited by imatinib. In addition to mast cell maturation and viability, c-kit tyrosine kinase receptor also links to degranulation of mast cells (Galli 1993). The present study showed that proliferation of cultured and synovial mast cells was inhibited by imatinib and the inhibitory effect was c-kit dependent. This study showed that the proliferation of synovial fibroblasts was inhibited by imatinib through the inhibition of PDGF-mediated signalling. As mast cells are involved in many aspects of the inflammatory reaction characteristic for RA (Nigrovic and

Lee 2005), the mast cell apoptosis in synovium could alleviate the inflammatory reaction. The proapoptotic effect of imatinib was attributed to its capacity to inhibit c-kit receptors on mast cells. Supporting this concept, mast-cell-activation-induced TNF-α secretion by synovial tissue explants was found to be inhibited by imatinib. Imatinib also inhibited proliferation of synovial fibroblasts stimulated by PDGF or serum. Typical features of RA SFB are enhanced production of matrix-degrading enzymes and cytokines and an excessive and invasive growth. As synovial fibroblasts are one central cell type directly contributing to joint destruction, the inhibition of SFB proliferation by imatinib may be beneficial in RA and may result in an antirheumatic effect. Next, it would be of interest to study the effects of imatinib on the MMP and cytokine production in SFB.

The synergistic inhibitory effect of imatinib on two types of synovial cells involved in the pathogenesis of RA, may well explain the recent clinical observations of antirheumatic activity of imatinib. However, a recent research paper describes and intriquing finding that imatinib inhibits also the M-CSF receptor, c-fms (Dewar et al. 2005). M-CSF appears to be central in the local activation, recruitment and survival of macrophage lineage cells at the site of inflammation (Hamilton 2002). Thus, M-CSF has been proposed as a potential factor for inhibition in order to yield an anti-inflammatory effect (Hamilton 2002). Thus, the capacity of imatinib to inhibit M-CSF receptor c-fms might also play a role in the possible antirheumatic activity.

A possible future approach to further elucidate the role of mast cells in RA could be the use of more selective c-kit inhibitors, which would leave the other members of the type III receptor tyrosine kinase family intact. The increased selectivity would most likely result in fewer side effects. However, it is impossible to predict whether the inhibition of c-kit would be sufficient to produce an antirheumatic effect as the inhibition of both PDGFR and c-fms potentially results in further benefits in treatment of RA, as discussed above.

In conclusion, results from preliminary clinical trials on the efficacy of imatinib in the treatment of RA are promising (Eklund and Joensuu 2003, Miyachi et al. 2003). The present study could, at least partially, provide a basis for the observed antirheumatic activity of imatinib. However, it remains to be seen whether the antirheumatic activity of imatinib can be confirmed in future clinical trials.

7.
SUMMARY AND CONCLUSIONS

In order to develop new therapies for RA, it is essential to increase the understanding of the mechanisms that contribute to the disease process. As RA is a chronic inflammatory disease of the joints, inflammatory mediators play an important role in the development of the disease. Several lines of evidence have indicated the involvement of mast cells in the pathogenesis of RA. One of the main findings of this study was that selective IgE-dependent activation of synovial mast cells results in production of two key cytokines, TNF-α and IL-1β, in rheumatoid synovial tissue explants. An increase in the secretion of anti-inflammatory IL-1Ra was also seen in response to synovial mast cell activation, but the relative increase in IL-1Ra was clearly smaller than that of IL-1β. Thus, mast cell activation was shown to be capable of contributing to the cytokine excess characteristic for RA and to shift the cytokine balance towards proinflammatory state. Based on the results of this study and previous findings by others, mast cells presumably play a role in the pathogenesis of RA. Thus mast cells can be seen as a potential target for antirheumatic therapy.

This study showed that imatinib and CMT-3 may represent novel compounds to inhibit mast cell function. Imatinib exerts a strong proapoptotic effect on cultured human mast cells and, importantly, on human synovial mast cells. The mechanism of action was attributed to the inhibition of the mast cell growth factor receptor c-kit. CMT-3 potently inhibits mast cell activation and cytokine production by mast cells, possibly by inhibiting PKC. CMT-3 induces also apoptosis in cultured mouse and human mast cells. Based on the results of this study and the previous findings, it can be concluded that further studies are warranted to determine the possible therapeutic efficacy of CMT-3 in arthritis

Destruction of the articular structures is a prominent feature of RA and it is pivotal in the development of disability. Synovial fibroblasts are the main effector cells in the joint destruction in RA, but they also contribute to the inflammatory process. These features of rheumatoid synovial fibroblasts make them an attractive target for antirheumatic therapy. This study showed that imatinib inhibits PDGF-stimulated proliferation of rheumatoid synovial fibroblasts, which might contribute to its antirheumatic effects.

Currently available DMARDs are limited by their ability to achieve early and sustained response, by their cumulative toxicity and by the lack of long-term remissions. The new biological therapies have greatly improved the treatment of severe RA. However, the biological drugs are extremely expensive, long-term remissions during the treatments are rare, and the disease tends to flare when the treatments

are discontinued. Furthermore, a significant proportion of patients fail to respond to these therapies, suggesting that additional important pathophysiological mechanisms may exist. This also reflects the need for new drug innovations for the treatment of the patients who do not benefit from current therapies. Imatinib represents one potential novel approach as it may exert antirheumatic activity by inhibiting mast cell activity and proliferation of synovial fibroblasts in rheumatoid synovium. The preliminar clinical findings on the efficacy of imatinib in the treatment of RA are consistent with this conclusion. Thus, imatinib could offer a new treatment strategy for those patients who fail the current antirheumatic therapy. Future clinical trials are needed to evaluate whether this approach is feasible.

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